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MATRIX METALLOPROTEINASES IN CRITICALLY ILL PATIENTS

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ACADEMIC DISSERTATION

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“Human beings don’t like things that are unexplained. We want the comfort and sense of safety that comes from predictability. Perhaps as we are evolved biological organisms, uncertainty is unsettling to us. And, in the scientific era, we assume a material understanding of causation. That’s what the idea of determinism represents in a simple, easy-to-grasp way. We want to be in control, to be able to manipulate nature to alleviate the problems that we face in a finite life in a finite world. We want our causes to be simple, real causes, and that is perhaps why the metaphor of the gene as the atom of causation in life is so easy to absorb, and its subtleties so easy to overlook. We are made very uneasy by things that are only probabilistic unless, as in coin-flipping, we can sense what’s going on. When we can’t see it, and causation is many-to-many, that is far too much for our minds to deal with easily. Yet that seems to be the reality of the world.”

-Weiss & Buchanan, 2013-

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on these original publications, referred to in the text by their Roman numerals.

I. Hästbacka J, Hynninen M, Kolho E, Pettilä V, Tervahartiala T, Sorsa T, Lauhio A: Collagenase 2/ matrix metalloproteinase 8 in critically ill patients with secondary peritonitis. *Shock* 2007; 27: 145-150

II. Lauhio A, Hästbacka J, Pettilä V, Tervahartiala T, Karlsson S, Varpula T, Varpula M, Ruokonen E, Sorsa T, Kolho E: Serum MMP-8, -9 and TIMP-1 in sepsis: High serum levels of MMP-8 and TIMP-1 are associated with fatal outcome in a multicentre, prospective cohort study. Hypothetical impact of tetracyclines. *Pharmacol Res* 2011; 64: 590-594

III. Hästbacka J, Tiainen M, Hynninen M, Kolho E, Tervahartiala T, Sorsa T, Lauhio A, Pettilä V: Serum matrix metalloproteinases in patients resuscitated from cardiac arrest. The association with therapeutic hypothermia. *Resuscitation* 2012; 83: 197-201

IV. Hästbacka J, Linko R, Tervahartiala T, Varpula T, Hovilehto S, Parviainen I, Sorsa T, Pettilä V: Serum MMP-8 and TIMP-1 in critically ill patients with acute respiratory failure: TIMP-1 is associated with increased 90-day mortality. Submitted

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LIST OF ABBREVIATIONS

AaDO₂ = Alveolar-arterial oxygen tension difference
AECC = American-European Consensus Conference
AKI = Acute kidney injury
ALI = Acute lung injury
AP = Acute pancreatitis
AP-1 = Activator protein-1
APACHE = Acute Physiology and Chronic Health Evaluation
ARDS = Acute respiratory distress syndrome
ARF = Acute respiratory failure
AT III = Antithrombin III
AUC = Area under the curve
BAL(F) = Broncho-alveolar lavage (fluid)
BBB = Blood-brain barrier
bFGF = Basic fibroblast growth factor
CAPD = Continuous ambulatory peritoneal dialysis
CINC = Cytokine-induced neutrophil chemoattractant
CMT = Chemically modified tetracycline
COPD = Chronic obstructive pulmonary disease
COX-2 = Cyclo-oxygenase-2
CPB = Cardiopulmonary bypass
CPR = Cardiopulmonary resuscitation
CRP = C-reactive protein
CSF = Cerebrospinal fluid
DAMP = Damage (danger)-associated molecular patterns
ECM = Extracellular matrix
EGF = Epidermal growth factor
ELISA = Enzyme-linked immunosorbent assay
EMMPRIN = Extracellular matrix metalloproteinase inducer
G-CSF = Granulocyte colony- stimulating factor
HMBG1 = High-mobility group box-1
ICAM-1 = Intercellular adhesion molecule-1
ICU = Intensive care unit
IFMA = Immunofluorometric assay
IFN- γ = Interferon-gamma
IGFBP = Insulin-like growth factor binding protein
IL = Interleukin
iNOS = Inducible nitric oxide synthase
IQR = Interquartile range
kDa = Kilodalton

LPS = Lipopolysaccharide
LR+ = Positive likelihood ratio
MCA = Medial cerebral artery
MCP-2 = Monocyte chemotactic protein-2
MIP-1 α = Macrophage inflammatory protein-1-alpha
MMP = Matrix metalloproteinase
MODS = Multiple organ dysfunction score
MOF = Multiple organ failure
MPO = Myeloperoxidase
MTH = Mild therapeutic hypothermia
NE = Neutrophil elastase
NF κ B = Nuclear factor kappaB
NGAL = Neutrophil gelatinase-associated lipocalin
NIV = Non-invasive ventilation
NOS = Nitric oxide synthase
PAMP = Pathogen-associated molecular patterns
PaO₂/FiO₂ = Partial pressure of arterial oxygen divided by fraction of inspired oxygen
PCWP = Pulmonary capillary wedge pressure
PDGF = Platelet-derived growth factor
PMN = Polymorphonuclear leukocytes
RAGE = Receptor for advanced glycation end-products
RECK = Reversion-inducing cysteine-rich protein with kazal motifs
RNS = Reactive nitrogen species
ROC curve = Receiver-operator characteristic curve
ROS = Reactive oxygen species
ROSC = Restoration of spontaneous circulation
SAP = Severe acute pancreatitis
SAPS = Simplified Acute Physiology Score
SIRS = Systemic inflammatory response syndrome
SLPI = Secretory leukocyte peptidase inhibitor
SOFA = Sequential Organ Failure Assessment
SuPAR = Soluble urokinase plasminogen activator receptor
TACE = TNF-alpha converting enzyme
TFPI = Tissue factor pathway inhibitor
TGF β = Transforming growth factor beta
TIMP = Tissue inhibitor of metalloproteinases
TLR = Toll-like receptor
TNF α = Tumour necrosis factor alpha
uPA = Urokinase-type plasminogen activator
VAP = Ventilator-associated pneumonia
VILI = Ventilator-induced lung injury
WT = Wild-type

ABSTRACT

AIMS

The systemic levels of matrix metalloproteinases (MMPs) -7, -8 and -9 and the tissue inhibitor of metalloproteinase-1 (TIMP-1) were investigated in critically ill patients with systemic inflammation of varying aetiologies. The study aimed to describe the presence of neutrophil-derived MMP-8 in the infected organ compartment and body fluids in a patient group with a severe infection. A further aim was to examine the association of neutrophil-derived MMPs -8 and -9 and their tissue inhibitor (TIMP-1) with outcome of critically ill patients. Finally, the effect of mild therapeutic hypothermia treatment on these variables was evaluated to determine their involvement in the therapeutic mechanisms of the treatment.

PATIENTS AND METHODS

The study population comprised 877 patients. In Study I, 15 ICU-treated adult patients with secondary peritonitis were prospectively included. Peritoneal fluid, blood and urine samples were collected simultaneously on inclusion to the study. MMP-8 levels were analysed from the samples by using immunofluorometric assay (IFMA). The serum and urinary levels of MMP-8 were compared with the peritoneal fluid levels. The serum levels were compared with those obtained from ten healthy volunteers. The collagenolytic activity of the peritoneal fluid was detected by SDS-polyacrylamide gel electrophoresis. The main collagenolytically active protein was identified by Western immunoblotting.

Study II was a sub-study of the observational multicentre FINNSEPSIS study where patients with severe sepsis or septic shock were prospectively included in 24 Finnish ICUs during a 4-month study period. The patient group of this study comprised 248 patients who consented to blood sample taking. Serum samples taken on admission to the study were analysed for MMP-8, MMP-9 and TIMP-1 levels by ELISA. The MMP and TIMP-1 levels were compared with those of ten healthy volunteers. Associations of MMPs -8 and -9 and TIMP-1 with ICU mortality were assessed by comparing the serum levels of survivors and non-survivors.

Study III was a retrospective laboratory analysis of 51 patients resuscitated from cardiac arrest. The patients were a subgroup of the Hypothermia After Cardiac Arrest study. Thirty of the patients had received mild therapeutic hypothermia treatment and 21 standard non-hypothermia treatment. Serum MMP-7, MMP-8, MMP-9

and TIMP-1 levels at 24 and 48 hours from restoration of spontaneous circulation (ROSC) were analysed. The serum levels of MMPs and TIMP-1 were compared between cardiac arrest patients and ten healthy volunteers. The association of hypothermia treatment was examined by comparing the serum MMP and TIMP-1 levels of hypothermia-treated patients during and after hypothermia with the levels of nonhypothermia-treated patients.

Study IV was a substudy of the FINNALI study conducted in 25 Finnish ICUs during an 8-week period. Patients with acute respiratory failure defined by the need for mechanical ventilation for more than 6 hours were included in the original study. Patients who consented to blood samples and who were not immunocompromised were included in this sub-study, which comprised 563 patients. MMP-8 and TIMP-1 were analysed by IFMA and ELISA, respectively, from blood samples taken on study admission and 48 hours thereafter. Association of MMP-8 and TIMP-1 with 90-day mortality and the discriminative power in predicting 90-day mortality were examined in all patients and in a subgroup of patients with acute lung injury or acute respiratory distress syndrome.

MAIN RESULTS

High levels of collagenase recognized as mainly neutrophil-type MMP-8 were detected in the peritoneal fluids of patients with secondary peritonitis. The median levels of MMP-8 in the sera and urine of the patients were elevated compared with healthy volunteers. Median MMP-8 levels were significantly lower in the serum and urine samples than in the peritoneal fluid, and the levels in different fluids did not intercorrelate.

Median MMP-8, MMP-9 and TIMP-1 levels were elevated in the serum of severe sepsis or septic shock patients compared with healthy controls. Higher median levels of MMP-8 ($p < 0.01$) and TIMP-1 ($p < 0.001$) were found in ICU non-survivors than in ICU survivors. MMP-9 levels were lower in non-survivors than in survivors ($p = 0.047$).

Systemic MMP-8 and MMP-9 were elevated in cardiac arrest patients relative to healthy controls. Patients who received hypothermia treatment had lower median MMP-9 levels during hypothermia than non-hypothermia-treated patients ($p < 0.001$).

Serum MMP-8 poorly predicted 90-day mortality of acute respiratory failure patients. Admission TIMP-1 levels were higher in non-survivors than in survivors ($p < 0.001$). TIMP-1 was an independent predictor of 90-day mortality, with a moderate discriminative power (AUC 0.633, 95% CI 0.580- 0.686). TIMP-1 was also associated with the severity of oxygenation disturbance. TIMP-1 levels were higher in the ALI/ARDS subgroup than in the whole cohort ($p < 0.01$).

CONCLUSIONS

The systemic levels of MMP-8 and MMP-9 are elevated in various groups of critically ill patients compared with healthy controls. Serum TIMP-1 increases in severe sepsis or septic shock patients, but reduced levels are seen after cardiac arrest compared with healthy controls. High levels of MMP-8 are present in the peritoneal fluid of patients with secondary peritonitis, and they greatly exceed those measured simultaneously in serum and urine. Systemic MMP-8 is associated with increased ICU mortality in patients with severe sepsis or septic shock, but not with long-term mortality. Among severe sepsis or septic shock patients, lower levels of MMP-9 are associated with increased ICU mortality. Elevated TIMP-1 is associated with outcome in patients with severe sepsis or septic shock and in patients with acute respiratory failure. TIMP-1 is a potentially useful biomarker for predicting 90-day mortality in acute respiratory failure patients. Serum MMP-9 levels may be down-regulated by mild therapeutic hypothermia treatment. This is one potential mechanism for how mild therapeutic hypothermia affects outcome of cardiac arrest patients.

KEY WORDS

Matrix metalloproteinase, tissue inhibitor of metalloproteinase-1, critical illness, systemic inflammation, severe infection, peritonitis, severe sepsis, acute respiratory failure, acute lung injury, post-cardiac arrest syndrome, therapeutic hypothermia, mortality

1. INTRODUCTION

Systemic inflammatory response syndrome (SIRS), defined clinically by leukocytosis or leukopenia, fever or hypothermia, tachypnoea and tachycardia, is present in most patients requiring intensive care (Bone et al. 1992, Sprung et al. 2006, Dulhunty et al. 2008). In the case of sepsis, SIRS is triggered by infection, but also non-infectious insults such as pancreatitis, trauma, massive haemorrhage, burns and ischaemia-reperfusion injury may induce the syndrome (Bone et al. 1992). Mortality rates of equal magnitude have been reported for infectious and non-infectious triggers of SIRS (Dulhunty et al. 2008). The inflammatory response is initiated by the mechanisms of innate immunity, by recognition of damage (or danger)-associated conserved molecular patterns (DAMPs), which include pathogen-associated molecular patterns (PAMPs) and alarmins. Alarmins are various markers of tissue injury that are released by cells in distress or cells undergoing necrotic death (Bianchi et al. 2007). Pattern recognition receptors located on polymorphonuclear leukocytes, lymphocytes and macrophages mediate signals to the nucleus to activate transcription of pro-inflammatory mediators (Bianchi et al. 2007). In SIRS, the extremely complex biological cascades that are activated during local compartmentalized inflammation are generalized and act in an uncontrolled manner in the whole body (Fry 2012). Activation of coagulation cascades, increased vascular permeability and loss of circulatory homeostasis lead to impaired tissue perfusion and oxygenation, organ dysfunction and ultimately to death of the patient. Although the amount of early organ dysfunction correlates well with outcome (Moreno et al. 1999), today's intensive care provides sophisticated methods to support dysfunctioning organs, and the majority of patients survive the initial shock phase (Hotchkiss et al. 2006). Subsequently, most patients recover after an appropriate dampening of the inflammation. However, at least partly depending on the degree of SIRS, some patients develop sustained organ dysfunction or failure, now the most common cause of mortality in critically ill patients (Knaus et al. 1985, Sprung et al. 2006).

A host of preclinically promising therapies designed to pharmacologically ameliorate the inflammatory response have failed to benefit SIRS patients in clinical studies in terms of improved survival, and therefore, the therapy principally remains supportive. In order to develop new therapies, it is important to further explore the pathophysiological mechanisms of local and generalized inflammation. One of the proposed reasons for disappointing results in the originally promising pharmacotherapies is that the spectrum of the therapies has been narrow in view of the complexity of the often parallel and redundant inflammatory cascades.

Furthermore, inhibiting the initiators of the cascades may come too late considering clinical reality because patients often have full-blown SIRS upon presentation. Therefore, as well as the initiators, it may be useful to further explore the effectors in the inflammatory cascade, neutrophil granulocytes being among the first-line players. Upon activation by pro-inflammatory stimuli, neutrophils release potent proteinases that, although necessary in eradication of the invading organism, have the capacity to cause direct collateral damage to tissues if the concentrations exceed those of their inhibitors (Owen et al. 1999). Among such proteinases are matrix metalloproteinases (MMPs) -8 and -9. Traditionally believed to play a role mainly in the processing of the extracellular matrix, they are now recognized as important tuners and amplifiers of inflammatory reactions.

MMPs participate in these reactions in almost all stages, beginning from chemotaxis and the transmigration of neutrophils from the circulation to the site of infection or tissue damage (Opdenakker et al. 2001, Vanlaere et al. 2009). Experimental evidence of the important roles of these enzymes in severe infection, sepsis and inflammation caused by non-infectious triggers is growing, but clinical studies are still limited. There are several classes of pharmacological MMP inhibitors, and interestingly, the familiar and relatively safe tetracycline group of antibiotics inhibit the expression and activity of MMPs by a mechanism independent from their antimicrobial functions (Hanemaaijer et al. 1997, Golub et al. 1998). In animal studies, the inflammatory response and subsequent organ dysfunction can often be alleviated or prevented by using pharmacological MMP inhibitors with a consequent survival benefit. These results may not, however, be directly translated to human patients due to differences between species, different timing of the inhibitor related to the triggering event and, especially considering sepsis, the incomplete equivalence between experimental models and human sepsis. Therefore, before moving on to clinical studies on MMP inhibition, more detailed information about the behaviour of these enzymes in association with critical illness is needed.

To evaluate the role of neutrophil-derived metalloproteinases in human SIRS, the studies included in this thesis investigated the systemic levels of these metalloproteinases and their regulators and inhibitors in different groups of critically ill patients. These groups included patients with severe infection, severe sepsis or septic shock, but also cardiac arrest patients to represent SIRS triggered by a different mechanism, namely ischaemia-reperfusion injury. To investigate the role of MMPs and their inhibitors in association with organ dysfunction, a group of patients with acute respiratory failure was included. The association of systemic MMP levels with outcome was examined, as was their usefulness as biomarkers in predicting mortality. In addition, the association of certain modes of therapy with the levels of MMPs and their inhibitors was evaluated.

ACTIVATOR EVENTS

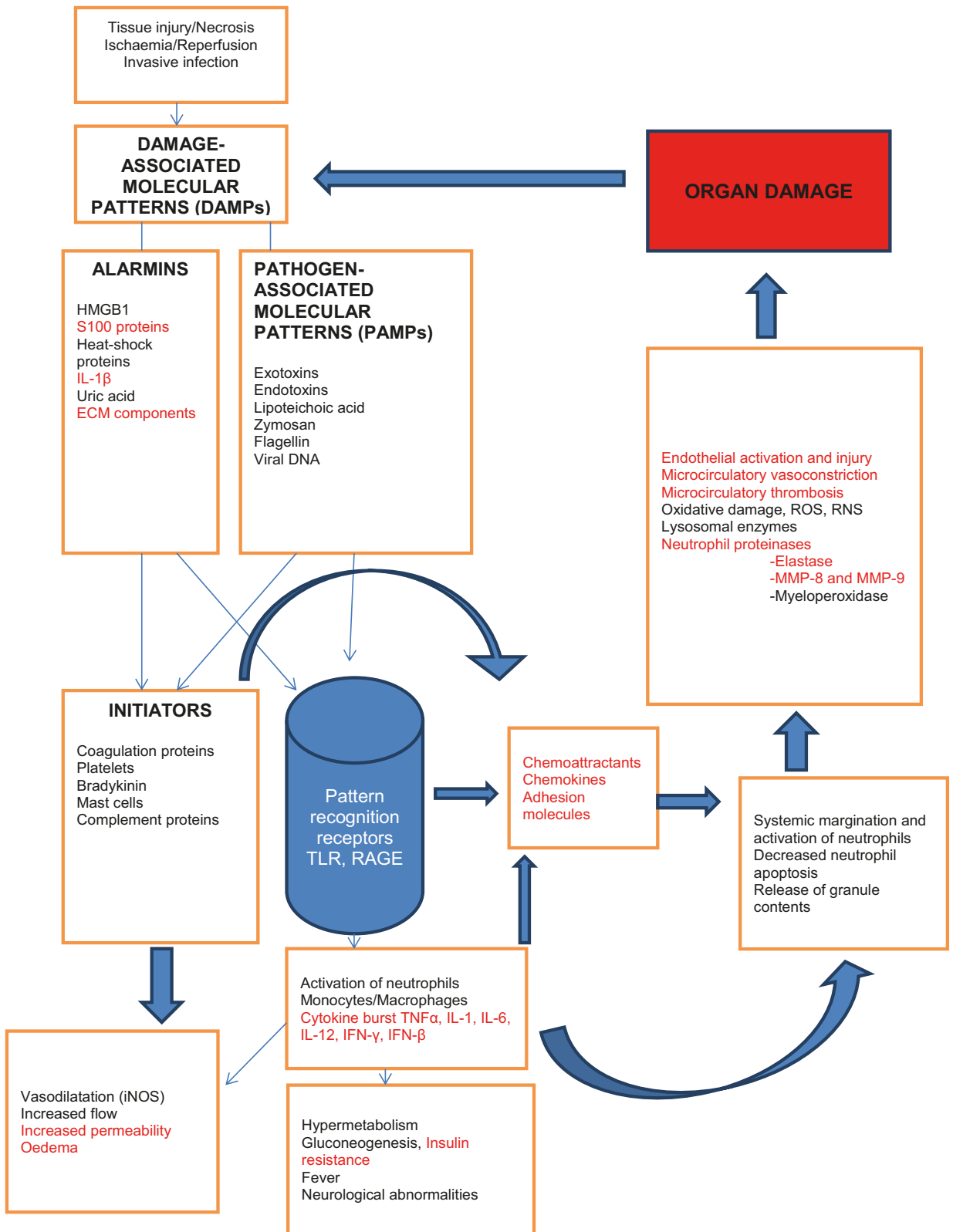


Figure 1. Simplified schematic representation of systemic inflammatory response activated by damage-associated molecular patterns (DAMPs). The activated cascades lead to clinical representations and ultimately to organ dysfunction, which may further amplify the cascade via additional development of DAMPs by tissue ischaemia and necrosis. The phases in which MMPs have been suggested to participate are highlighted in red. Modified from Vanlaere et al. 2009 and Fry 2012.

2. REVIEW OF THE LITERATURE

2.1. EXTRACELLULAR MATRIX, NEUTROPHIL-DERIVED MATRIX METALLOPROTEINASES AND THEIR REGULATION

2.1.1. EXTRACELLULAR MATRIX (ECM)

The extracellular matrix is an important playground of matrix metalloproteinases. The ECM is an acellular component of tissue consisting of interstitial collagens I, II and III, proteoglycans, fibronectin, and basement membrane components collagen IV, laminin and entactin. It also contains hyaluronan, which participates in the regulation of cell adhesion, migration and signalling (Stamenkovic 2003). ECM is essential in tissue architecture and homeostasis, but its functions reach far beyond being the passive structural component of tissues, and it is an important mediator of cell-cell interactions and cell signalling. The ECM is a reservoir of resting cytokines, proteases and growth factors that are liberated from this mesh upon breakdown of the matrix proteins (Stamenkovic 2003). The intact ECM mediates signals affecting cell survival, and damage to its structure may initiate processes leading to cell death. A phenomenon called anoikis-like cell death refers to a process where interaction between epithelial cells and ECM is disrupted, a survival signal from intact interaction is lost and cellular apoptotic mechanisms are activated (Frisch et al. 1994). Attachment of the epithelial cells to the basement membranes seems to be of particular importance because disruption of this contact by inactivating the integrins promotes apoptosis (Boudreau et al. 1995).

During various physiological and pathophysiological processes the ECM is subject to continuous remodelling. MMPs are central players in these processes, which include morphogenesis, angiogenesis, growth, wound healing and reproduction-associated processes, but also disease processes such as tumour invasion and metastasis, acute and chronic inflammation and atherosclerosis (Vanlaere et al. 2009, Rodriguez et al. 2010). In acute inflammation, the ECM and basement membranes are degraded as leukocytes migrate from the circulation to the site of inflammation and release potent proteases, such as MMPs. As MMPs are together able to digest proteolytically virtually all components of the ECM, this matrix-degrading function was traditionally perceived as their main function in inflammatory processes. However, recent work has elucidated that the most important functions of MMPs include mobilizing and activating cytokines and

growth factors from the ECM (Rodriguez et al. 2010) and working as tuners and amplifiers of immune functions (Opdenakker et al. 2001).

2.1.2. NEUTROPHILS

Neutrophils are the first-line effector cells in innate immune defence against invading microbial organisms (Marshall 2005). They express pattern recognition receptors, such as Toll-like receptors, that recognize conserved molecular patterns found on microbes (Hayashi et al. 2003). Recognition of these patterns leads to activation of circulating neutrophils to express L-selectins, which recognize adhesion molecules and selectins on the activated endothelium, thus facilitating adherence to the capillary endothelium (Marshall 2005). Neutrophils are directed to the site of injury by fast chemoattraction by CXC chemokines, e.g. interleukin-8 (IL-8), the most abundant chemokine in humans. Activation of the chemokine receptors leads to migration through the capillary wall towards a chemokine gradient, release of intracellular granules and initiation of the respiratory burst. During the migration and killing of the microbes neutrophils produce reactive oxygen species and nitrogen molecules and release proteolytic enzymes (Marshall 2005). The latter include serine proteinases such as elastase, cathepsin G, urokinase-type plasminogen activator and myeloperoxidase and MMP-8 and -9 (Owen et al. 1999). These proteinases have the potential to cause extensive collateral damage to the surrounding tissues (Marshall 2005). On the other hand, deficiency of proteinases involved in the migration process leads to an increase in life-threatening infections (Gallin et al. 1985).

After phagocytosis and bacterial killing neutrophils undergo self-programmed apoptosis and are phagocytosed by macrophages (Savill et al. 1989). Subsequently, anti-inflammatory response is initiated in the macrophages with suppression of tumour necrosis factor- α (TNF- α) and increased secretion of transforming growth factor- β (TGF- β) and IL-10, and the inflammatory response is tuned down (Savill et al. 2002). In sepsis, neutrophil apoptosis is significantly reduced (Keel et al. 1997). Pro-inflammatory cytokines TNF- α and IL-1 and IL-12 contribute to the reduced spontaneous apoptosis, opposed by the anti-inflammatory IL-10 (Keel et al. 1997). The decreased neutrophil apoptosis may be detrimental by several mechanisms, with continuing release of harmful mediators and subsequent tissue injury. It may also lead to impaired resistance to secondary infections. In a recent study, those trauma patients with increased polymorphonuclear (PMN) leukocyte apoptosis in the early phase developed significantly less SIRS symptoms as well as less infections (Morrison et al. 2012).

2.1.3. MATRIX METALLOPROTEINASE FAMILY

As neutrophils secrete MMP-8 and MMP-9, there is a large group of MMPs released from other cell types. The first MMP, a collagenase, was described in 1962 by Gros and Lapière in an involuting tail of a metamorphosing tadpole (Gros and Lapière 1962). Since then, at least 25 members of the MMP family have been discovered (Van Lint et al. 2006). MMPs are genetically distinct and structurally related zinc- and calcium-dependent endopeptidases (Sternlicht et al. 2001). MMPs have substantial overlap in their substrates, and together they are able to digest all components of the ECM (Sternlicht et al. 2001). On the basis of their preferred substrate specificity MMPs can be divided into six groups: collagenases (MMP-1, -8 and -13) degrade interstitial collagen; gelatinases (MMP-2 and -9) degrade denatured collagen, gelatin; stromelysins (MMP-3 and -10 and -11) digest ECM components and activate other MMPs; matrilysins (MMP-7 and -26) degrade ECM components and cell surface molecules; membrane-type MMPs (MMP-14, -15, -16, -17, -24 and -25), and others (reviewed in Visse 2003). Neutrophil-derived MMP-8 and -9 are described in detail below.

2.1.4. REGULATION OF MATRIX METALLOPROTEINASES

MMPs are regulated at several levels, namely transcription, activation of the pro-enzyme and inhibition of the active enzyme. Most cell types do not express MMPs in the resting state, but certain stimuli such as pro-inflammatory cytokines activate the transcription. MMPs are secreted as inactive zymogens that need to be activated—for most MMPs, this occurs extracellularly. The activation involves a “cysteine switch” that requires displacing of a sulfhydryl group of a cysteine residue at the catalytic site and thus exposing the zinc needed for the catalytic actions (Springman et al. 1990). Subsequent autocatalysis or cleavage by other active MMPs leads to formation of the active proteinase (reviewed in Visse 2003). MMP activators include serine proteases, oxidized glutathione, other MMPs (Visse 2003) and reactive oxygen (Peppin et al. 1986) and nitrogen species (Gu et al. 2002). In the body fluids, the most important inhibitor is the non-specific plasma antiprotease α 2-macroglobulin, which binds to MMPs covalently (Sottrup-Jensen et al. 1989). At the tissue level, MMPs are inhibited by non-covalently binding to tissue inhibitors of metalloproteinases (TIMPs) (reviewed in Visse 2003). Unbound, MMPs may also undergo spontaneous degradation by autocatalysis (Yan et al. 2001). MMPs may avoid inhibition by TIMPs by cell membrane localization (Owen et al. 2004), but they can be inhibited also on the cell surface. For example, a cell surface receptor RECK (reversion-inducing cysteine-rich protein with kazal motifs) is a cell surface MMP inhibitor (Oh et al. 2001). MMP inhibition is concentration-dependent because TIMPs bind MMPs in

a 1:1 molecular ratio. The inhibition by TIMPs as well as plasma proteinases may be circumvented by leukocytes locally by creating a plasma-free microenvironment where pro-MMP is secreted and activated for facilitating transmigration through basement membranes (Delclaux et al. 1996). This is beneficial when controlled, but in case of overwhelming inflammation, when the amount of metalloproteinases may exceed the amount of their inhibitors, they have the capacity to cause damage to the tissues, especially to the ECM structures.

2.1.5. MATRIX METALLOPROTEINASE-8 (MMP-8)

Matrix metalloproteinase-8, also known as collagenase-2, belongs to the group of interstitial collagenases. It was first described in 1968 by Lazarus et al. as a collagenolytic proteinase in PMN and purified from PMN in 1986 by Hasty et al. who also sequenced this PMN-secreted form (Hasty et al. 1990). Although MMP-8 can cleave collagens I, II and III, its preferred substrate is collagen I (Hasty et al. 1987), and it is the only PMN-derived proteinase able to digest type I collagen (Owen et al. 1999).

Traditionally, it was believed that MMP-8 is not synthesized *de novo* by mature PMN because the synthesis occurs predominantly at the myelocyte stage of maturing neutrophils (Cowland et al. 1999). However, also mature and activated neutrophils have been found to express MMP-8 mRNA (Cole et al. 1995). The latent 95 kDa proenzyme is stored in specific granules of PMN leukocytes (Murphy et al. 1977). Human peripheral blood neutrophils contain about 60 ng MMP-8/ 10^6 cells and they release small amounts when unstimulated (Owen et al. 2004). When the cells are activated by inflammatory stimuli, they release about 15-20% of their stored enzyme as a soluble proteinase (Owen et al. 2004). MMP-8 is also located bound to the cell membrane of activated neutrophils, where it is proteolytically active and resistant to tissue inhibitors (Owen et al. 2004). When not bound to the membranes, the half-life of soluble MMP-8 in 37°C is 7.5 hours (Owen et al. 2004). MMP-8 is synthesized also by various other cell types, examples of which are provided in Table 1. The synthesis is upregulated by pro-inflammatory cytokines. Substances involved in the regulation are shown in Table 1.

2. REVIEW OF THE LITERATURE

Table 1. Cell types expressing MMP-8 and MMP-9, regulators of synthesis, substances involved in enzyme activation and enzyme substrates.

	Cell type (reference)	Regulators of synthesis	Enzyme activated by	Substrates cleaved
MMP-8	-Neutrophil granulocytes (<i>Hasty et al. 1986</i>)	- ↑ TNF- α (<i>Hanemaaijer et al. 1997</i>)	-Trypsin, chymotrypsin, pancreatic kallikrein, cathepsin G (<i>Knäuper et al. 1990</i>)	-Collagen I, II, III (<i>Hasty et al. 1987</i>)
	-Chondrocytes (<i>Cole et al. 1996</i>)	- ↑ IL-1 β (<i>Chubinskaya et al. 1996</i>)		-Bradykinin, angiotensin I, substance P (<i>Diekmann et al. 1994</i>)
	-Rheumatoid synovial fibroblasts (<i>Hanemaaijer et al. 1997</i>)	- ↑ IL-6 (<i>Wahlgren et al. 2001</i>)	-MMP-3 (<i>Knäuper et al. 1993</i>)	- α 1-antitrypsin, α 1- antichymotrypsin (<i>Michaelis et al. 1990</i> , <i>Desrochers et al. 1992</i>)
	-Activated macrophages (<i>Herman et al. 2001</i>)	- ↓ TGF- β (<i>Palosaari et al. 2000</i>)	-MMP-7 <i>Balbin et al. 1998</i>)	- α 2-macroglobulin (<i>Sottrup-Jensen et al. 1989</i>)
	-Smooth muscle cells (<i>Herman et al. 2001</i>)		-Tryptase (<i>Gruber et al. 1988</i>)	-MIP-1 α (<i>Quintero et al. 2010</i>)
	-Bronchial epithelial cells (<i>Prikk et al. 2001</i>)		-Reactive oxygen species (<i>Saari et al. 1992</i> , <i>Claesson et al. 1996</i>)	-IL-10 (<i>Garcia-Prieto et al. 2010</i>)
	-Endothelial cells (<i>Hanemaaijer et al. 1997</i>)		-Peroxynitrite (<i>Okamoto et al. 2001</i>)	-Chemokines (<i>Van den Steen et al. 2003</i>)
	-Odontoblasts (<i>Palosaari et al. 2000</i>)			
	-Lymphocytes (<i>Lindberg et al. 2006</i>)			
	-Plasma cells (<i>Wahlgren et al. 2001</i>)			

	Cell type (reference)	Regulators of synthesis	Enzyme activated by	Substrates cleaved
MMP-9	<ul style="list-style-type: none"> -Neutrophil granulocytes (Murphy et al. 1989) -Mononuclear phagocytes (Welgus et al. 1990) -Eosinophils (Ohno et al. 1997) -Macrophages (Hibbs et al. 1987) -T-lymphocytes (Leppert et al. 1996) -B-lymphocytes (Trocmé et al. 1998) -NK cells (Kitson et al. 1998) - Fibroblasts (Wilhelm et al. 1989) -Vascular endothelial cells (Renckers et al. 2006) -Smooth muscle cells (Renckers et al. 2006) -Platelets (Fernandez-Patron et al. 1999) -Mesothelial cells (Marshall et al. 1993) -Neurons, astrocytes, oligodendrocytes, microglia (Conant et al. 1999, Rivera et al. 2002, Rosenberg 2001) - Amnion epithelial cells (Lehtovirta et al. 1994) 	<ul style="list-style-type: none"> - ↑ TNF-α (Sarén et al. 1996, Unemori et al. 1991) - ↑ IL-1β (Sarén et al. 1996, Unemori 1991) - ↑ IL-2 (Kitson et al. 1998) - ↑ TGF- β (Wahl et al. 1993) - ↑ LPS (Welgus et al. 1990) - ↓ IL-10 (Mostafa Mtairag et al. 2001) - ↓ IFN-γ, IFN-β (Shapiro et al. 1990, Sarén et al. 1996, Leppert et al. 1996) - ↓ IL-4 (Lacraz et al. 1992) - ↓ Glucocorticoids (Aljada et al. 2001) 	<ul style="list-style-type: none"> -Nitric oxide (Gu et al. 2002) -Reactive nitrogen species (Okamoto et al. 2001) -Reactive oxygen species (Peppin et al. 1986) -Trypsin (Sorsa et al. 1997) -Stromelysin-1, MMP-2 (Ogata et al. 1992, Fridman et al. 1995) -MMP-7 (Balbin et al. 1998) -Plasmin (Baramova et al. 1997, Makowski et al. 1998) -PMN elastase (Ferry et al. 1997) -Bacterial products (Oggioni et al. 2003) 	<ul style="list-style-type: none"> - Gelatin, type IV collagen -Type V collagen (Murphy et al. 1977) - Collagen VII, X, elastin (Senior et al. 1991) -Laminin (Owen et al. 1999) -Aggrecan (Fosang et al. 1992) -IL-1β (Scönbeck et al. 1998, Ito et al. 1996) -IL-8 (Van den Steen et al. 2000) -Other chemokines (Van den Steen et al. 2003) -TNF-α (release) (Gearing et al. 1994) -TGF β (Yu et al. 2000) -IL-2 receptor (Sheu et al. 2001) - α1-proteinase inhibitor (Desrocher et al. 1992, Liu et al. 2000) -Big endothelin (Fernández-Patron et al. 2001)

The table gives examples of the origin, regulation of synthesis and activity as well as some substrates of MMP-8 and MMP-9. Different cell types respond to different regulators of synthesis, but for simplicity these differences are not indicated in the table. MMP= matrix metalloproteinase; TNF- α = tumour necrosis factor- α ; IL= interleukin; TGF- β = transforming growth factor- β ; MIP-1 α = macrophage inflammatory protein; LPS= lipopolysaccharide

Like other MMPs, MMP-8 is secreted as an inactive pro-enzyme that needs to be activated. Examples of substances involved in MMP-8 activation are presented in Table 1. The enzyme is inactivated by EDTA, cysteine and reduced glutathione and also by omitting calcium (Lazarus et al. 1968). The physiological functions of MMP-8 are not restricted to ECM collagen degradation, and considering the variety of inflammatory mediators as its substrates, it is likely to play an important role

in inflammatory reactions. Solan et al. (2012) indicated recently that MMP-8 can activate nuclear transcription factor nuclear factor kappaB (NFkB) directly, thus leading to the pro-inflammatory phenotype in macrophages with increased cytokine and chemokine production. Examples of MMP-8 substrates are presented in Table 1. It can cleave vasoactive substances, inactivate proteolytically serine protease inhibitors and activate and inactivate chemokines. MMP-8 is considered important in neutrophil chemotaxis (Tester et al. 2007). Based on studies on mice genetically lacking MMP-8, a characteristic dual role in inflammation seems evident: lack of the enzyme produces delayed accumulation of neutrophils to the site of inflammation, but also a delayed clearance of the neutrophils and therefore delayed healing. This may be due to decreased neutrophil apoptosis (García-Prieto et al. 2010, Balbin et al. 2003). For example, mice genetically lacking MMP-8 have delayed wound healing and prolonged inflammation characterized by delayed but prolonged accumulation of neutrophils due to delayed neutrophil apoptosis (Gutiérrez-Fernández et al. 2007). The delayed accumulation of neutrophils in the absence of MMP-8 suggests its important role in neutrophil trafficking and/or chemotaxis.

Delayed wound healing has been described also in rats overexpressing MMP-8 in skin fibroblasts. Overexpression of MMP-8 was accompanied by decreased amounts of myeloperoxidase at later stages of healing, which in turn suggests that MMP-8 may have a role in the dampening of neutrophil response. A decreased collagen content and impaired tensile strength was seen in the wounds, suggesting that high tissue levels of MMP-8 delay proper healing (Danielsen et al. 2011).

MMP-8 has a pathophysiological role in chronic inflammatory diseases such as rheumatoid arthritis (Matsuki et al. 1996), osteoarthritis (Cole et al. 1995), cystic fibrosis (Power et al. 1994), periodontal disease (Lee et al. 1995), chronic skin wounds (Nwomeh et al. 1999), and atherosclerosis (Pradhan-Palikhe et al. 2010). It has also been shown to promote tumour invasion and metastasis in several studies (reviewed in Van Lint et al. 2006).

2.1.6. MATRIX METALLOPROTEINASE-9 (MMP-9)

MMP-9 is another metalloproteinase secreted by neutrophils (Murphy et al. 1989). It was first described in human leukocytes in 1974 by Sopata and Danciewicz. In the earlier literature, MMP-9 has been referred to as type IV collagenase, type V collagenase, 92-kDa collagenase and gelatinase B. Like MMP-8, it is mostly synthesized in maturing neutrophils, but synthesis is also possible in mature neutrophils (Nagaoka et al. 2000). It is stored in tertiary granules of PMN (Borregaard et al. 2001), and almost immediate degranulation occurs after neutrophil activation with IL-8 (Masure et al. 1991, Pugin et al. 1999^a), lipopolysaccharide (LPS) stimulation

(Pugin et al. 1999^a), TNF- α , G-CSF (granulocyte colony stimulating factor), phytohaemagglutinin (Pugin et al. 1999^a) or tissue plasminogen activator (Cuadrado et al. 2008). It is also expressed in various other cell types (Table 1). In contrast to neutrophils, MMP-9 is not stored in most of the other cells and its constitutive expression is limited. Synthesis is upregulated at least *via* nuclear transcription factor AP-1 (Speidl et al. 2004). The LPS-induced expression and activity is further potentiated by catecholamines in human monocytes, probably via a β 1-receptor mediated mechanism (Speidl et al. 2004). *In vivo*, MMP-9 is upregulated very fast after LPS challenge (Pugin et al. 1999^a, Paemen et al. 1997). The synthesis is suppressed by anti-inflammatory cytokines, which also upregulate the expression of TIMP-1 in many cell types. Glucocorticoids suppress MMP synthesis; for example, hydrocortisone suppresses plasma MMP-9 levels of healthy subjects within 1 hour of a single intravenous dose of 100 mg (Aljada et al. 2001). Dexamethasone does not block the degranulation of MMP-9 from neutrophils (Pugin et al. 1999^a), indicating that the suppression occurs at the level of transcription.

MMP-9 is secreted in several forms: as a 92 kDa pro-enzyme, as a 130 kDa complex with neutrophil gelatinase-associated lipocalin (NGAL) (Yan et al. 2001) and as a 200 kDa homodimer (Opdenakker et al. 2001^b). NGAL is thought to protect MMP-9 from degradation and to help preserve its enzymatic activity (Yan et al. 2001). Functionally, MMP-9 is a gelatinase and a type IV collagenase that is able to cleave collagen after an initial cleavage by collagenases, and native type IV collagen, which is a major component in basement membranes. It also digests various other components of the ECM (Table 1). Like other MMPs, MMP-9 needs to be activated to exert its functions. Activators include serine proteases, reactive oxygen and nitrogen species and other MMPs (Table 1). Also certain virulent strains of *Streptococcus pneumoniae* produce proteases that are able to cleave and thus activate MMP-9 (Oggioni et al. 2003). MMP-9 is active at physiological pH and temperature (Fasciglione et al. 2000). At the tissue level, MMP-9 is rapidly inactivated by TIMPs and proteolytic self-degradation (Yan et al. 2001).

MMP-9 has a multitude of functions at several stages of acute inflammation. It facilitates neutrophil transmigration across basement membranes in response to chemoattractant stimulation (Delclaux et al. 1996). It creates positive and negative feedback loops in processing cytokines; it cleaves interleukin 1 β precursor to the biologically active form (Schönbeck et al. 1998) and also degrades active interleukin-1 β (Ito et al. 1996). It processes interleukin-8, the most powerful neutrophil-activating chemokine, to a 10-fold more potent form (Van den Steen et al. 2000), processes other chemokines (Van den Steen et al. 2003), promotes TNF- α release (Gearing et al. 1994) and activates TGF- β (Yu et al. 2000). It also cleaves and inactivates IL-2 receptor, resulting in inhibition of T-cell proliferation (Sheu et al. 2001). It may also potentiate the function of other proteinases because it is

able to inactivate α 1-proteinase inhibitor, which is a major inhibitor of neutrophil elastase (Liu et al. 2000). By cleaving endothelin to its vasoactive form, endothelin-1, it stimulates its own release from neutrophils, creating a positive feedback loop, and promotes enhanced adhesion (Fernandez-Patron et al. 2001). Finally, MMP-9 may also contribute to the termination of inflammation by promoting neutrophil apoptosis because genetically MMP-9- deficient mice had at least transiently impaired neutrophil apoptosis in an experimental peritonitis model (Kolazkowska et al. 2009).

2.1.7. MATRIX METALLOPROTEINASE-7 (MMP-7)

MMP-7, also known as matrilysin or PUMP-1, is the smallest and structurally simplest of the MMPs, with a molecular weight of 28 kDa (Wilson et al. 1996). It is expressed constitutively by epithelial cells (Wilson et al. 1996) such as glandular epithelial cells of the mammary gland, pancreas, parotid gland, liver and peribronchial glands (Saarialho-Kere et al. 1995). MMP-7 is also produced by monocytes (Busiek et al. 1992).

It can cleave laminin and entactin (Wilson et al. 1996, Sires et al. 1993) and various other ECM components (Imai et al. 1995), insulin, transferrin, serpins (Sires et al. 1994), pro-uPa and uPa (Wilson et al. 1996). MMP-7 activates both MMP-8 (Balbin et al. 1998) and MMP-9 (Imai et al. 1995). It seems important in cleaving cell membrane proteins to their soluble forms, e.g. cell membrane bound TNF- α precursor to its active, soluble form (Gearing et al. 1994). Another membrane-bound protein, Fas-ligand (FasL), is cleaved into soluble FasL by MMP-7 to promote apoptosis by binding to its receptor on epithelial cells (Powell et al. 1999). In the intestine, MMP-7 is found in the Paneth cells in the crypts of small intestine (Wilson et al. 1995), where it activates and liberates directly microbicidal α -defensins (Wilson et al. 1999). Its expression is upregulated by adherent bacteria and pro-inflammatory cytokines (López-Boado et al. 2000). MMP-7 is important in epithelial repair and migration; for example, in airway injury the epithelial cells cannot migrate in the absence of MMP-7 (Dunsmore et al. 1998). Similar importance of MMP-7 in cell migration is seen in gastric epithelial cells in the presence of *Helicobacter pylori* infection (Wroblewski et al. 2003). Impaired transepithelial neutrophil influx from the interstitium to the alveoli is detected in mice lacking MMP-7 due to the absence of MMP-7-driven shedding of syndecan-1 from the cell surfaces (Li et al. 2002).

2.1.8. TISSUE INHIBITOR OF METALLOPROTEINASES-1 (TIMP-1)

The first TIMP discovered, now known as TIMP-1, was found in the 1970s in serum, and its ability to inhibit collagenases was recognized (Woolley et al. 1975). Four TIMPs have been identified to date in humans. TIMPs are small, stable proteins that inhibit MMPs by forming non-covalent bonds with 1:1 stoichiometry (reviewed in Lambert et al. 2004). TIMP-1 and TIMP-2 inhibit many MMPs, but TIMP-2 has a special affinity for MMP-2 (Howard et al. 1991). TIMP-2 inhibits also MMP-9 more effectively than TIMP-1 (Howard et al. 1991), but TIMP-1 prefers to form complexes with the pro-form of MMP-9 (Wilhelm et al. 1989). TIMP-1 is expressed in a variety of cells, with the exception of neutrophils, and is present in body fluids and most tissues (reviewed in Lambert et al. 2004). Regarding inflammatory situations, TIMP-1 is induced by several growth factors (reviewed in Lambert et al. 2004) and cytokines such as IL-1 β (Marshall et al. 1993), TNF- α (Marshall et al. 1993), IL-6 (Lacraz et al. 1992), IL-10 (Mostafa Mtairag et al. 2001) and bacterial LPS (Pagenstecher et al. 2000). Catecholamines upregulate its expression (Speidl et al. 2004).

The regulation by cytokines most likely depends on cell type because IL-1 β , TNF- α and LPS may also downregulate the synthesis (Yao et al. 1997). The synthesis is in part coordinately regulated with MMP expression, but certain regulators have an opposite effect. An example is TGF- β , which suppresses the expression of collagenases and increases TIMP-1 production, at least in certain cell types (Overall et al. 1994). TIMP-1 is inactivated by neutrophil elastase (Okada et al. 1988), myeloperoxidase (Wang et al. 2007) and peroxynitrite, a reactive nitrogen species (Frears et al. 1996), all released from neutrophils during inflammation. Functionally, TIMP-1 has various effects on neutrophils. It activates neutrophils, potentiates their respiratory burst, protects them from apoptosis and inhibits their transmigration across basement membranes (Delclaux et al. 1996, Chromek et al. 2004). TIMP-1 protects certain other cell types from apoptosis in a mechanism independent of MMP inhibition (Guedez et al. 1998).

2.2. MMP- 8, MMP-9 AND TIMP-1 IN SEVERE INFECTION AND SEPSIS

2.2.1 SEVERE INFECTION

Altered levels of MMP-8 and -9 have been shown in association with severe infections, where they have both anti- and pro-inflammatory functions. Clinical studies are few and small, with the exception of pulmonary infections, which are described in association with acute lung injury below.

2.2.1.1. Meningitis

Neisseria meningitidis can induce MMP-8 and it is in a key role in the associated permeability changes in the cerebral microvasculature by cleaving the tight intercellular junction protein occludin (Schubert-Unkmeir et al. 2010). Also MMP-9 induces the increased permeability and breakdown of the blood-brain barrier during experimental bacterial meningitis (Paul et al. 1998). The breakdown and permeability changes and subsequent elevated intracranial pressure can be inhibited by an MMP inhibitor (Paul et al. 1998). Thus, MMPs -8 and -9 may have pathophysiological relevance in bacterial meningitis. Experimental infection with intracisternal *Streptococcus pneumoniae* causes increased synthesis of MMPs -3, -7, -8 and -9 in the brain, and by using a broad spectrum MMP inhibitor neuronal damage could be prevented (Leib et al. 2000). In another study, a broad spectrum MMP inhibitor diminished cortical damage and hippocampal apoptosis as well as clinical symptoms, mortality and post-infection learning disturbances. Importantly, these effects were visible also with an inhibitor administered 18 hours post-infection together with the first dose of antibiotics, a situation resembling clinical reality (Leib et al. 2001). However, the inhibitor used in these studies inhibits also TACE, an enzyme activator of TNF- α , which is a key regulator of cerebral inflammation as well as a powerful MMP inducer (Leib et al. 2001). The same applies to doxycycline, which was administered 18 hours post-infection to rats with experimental pneumococcal meningitis as an adjuvant therapy with ceftriaxone. With doxycycline mortality, damage to the brain and hearing organs were significantly reduced, despite an *in vitro* antagonism of doxycycline and ceftriaxone demonstrated in the same study (Meli et al. 2006). The cellular origin of MMP-8 in bacterial meningitis has been suggested to be predominantly other cells than neutrophils (Lindberg et al. 2006). In clinical studies, elevated levels of MMP-9 (Paul et al. 1998, Leppert et al. 2000, Leib et al. 2000), MMP-8 and TIMP-1 (Leppert et al. 2000) are present in the cerebrospinal fluid of patients with bacterial meningitis. Leppert et al. also reported an association of high MMP-9 levels with neurological sequelae, but the study was methodologically compromised.

2.2.1.2. Peritonitis

In experimental mouse peritonitis, a specific time profile for MMP-9 has been described (Kolaczowska 2008). MMP-9 is initially produced by mast cells and peritoneal macrophages, followed by an intense neutrophil infiltration and MMP-9 release with a plateau at 2-8 hours (Kolaczowska et al. 2008). MMP-9 is also produced by peritoneal mesothelial cells (Marshall et al. 1993). MMP-9 seems to be especially important in neutrophil transmigration into the peritoneal cavity

because MMP-9-deficient mice have diminished peritoneal neutrophil influx after intraperitoneal *Escherichia coli* administration. However, in the same study in the distant organs the neutrophils were more abundant in knock-out mice than in the wild-type (WT) controls. In that study, the MMP-9-deficient mice had diminished bacterial clearance and more severe distant organ damage than the WT controls (Renckers et al. 2006). This suggests a protective role of MMP-9 in peritonitis. Evidence of MMP involvement in human peritonitis comes from studies on patients on chronic ambulatory peritoneal dialysis (CAPD). MMP-9 levels and activity are increased in dialysate fluids of patients with CAPD-associated peritoneal infection compared with non-infected CAPD patients, and then diminishing at the recovery phase at 15-30 days after onset. A simultaneous increase in TIMP-1 levels has also been detected (Fukudome et al. 2001). Here, the detection of MMP-9 in the infected compartment has led to development of diagnostic tools. A MMP-9 antibody-based rapid test kit has proven to detect bacterial peritonitis in peritoneal dialysis patients with high sensitivity (96.1%) and specificity (89.5%) (Ro et al. 2004). In addition to diagnostics, it may have pathophysiological importance, because at least experimental studies suggest that MMP-9 present in intestinal anastomoses contributes to the weakening of anastomotic strength on the 3rd post-surgical day. Moreover, the anastomotic strength can be increased by using an MMP inhibitor (Syk et al. 2001, de Hingh et al. 2002). An evident increase in the MMP-9 content of anastomotic regions was seen on the 3rd postoperative day when the animals were operated on under induced peritonitis conditions and they had a simultaneous weakening of anastomotic strength compared with controls operated on without peritonitis (de Hingh et al. 2003). This association was, however, transient and limited.

2.2.1.3. Other infections

MMP-8 is elevated in amniotic infection and may be relevant in preterm rupture of fetal membranes (Maymon et al. 1999).

2.2.2. SEPSIS AND SEPTIC SHOCK

In sepsis, a multitude of inflammatory mediators participate in the inflammatory response in an extremely complex manner. MMPs play a role in almost all stages of acute inflammation (Vanlaere et al. 2009). In Figure 1, the pathophysiological cascades associated with SIRS are shown and the possible stages for MMP involvement are indicated. Figure 2 illustrates some of the postulated roles of MMPs in local inflammation.

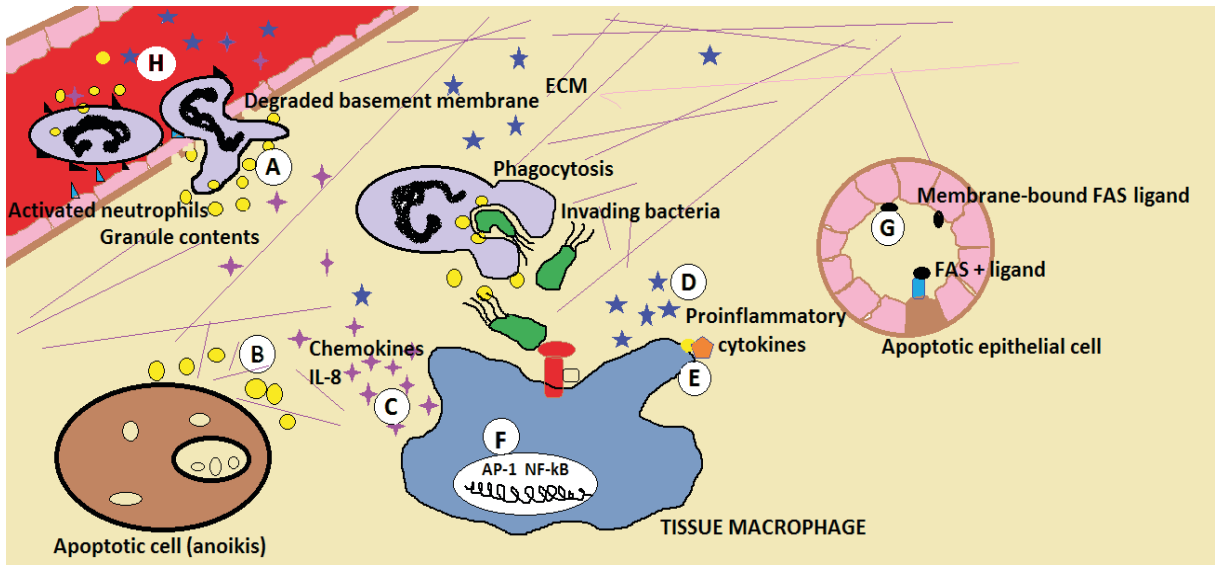


Figure 2. Localized inflammation. Bacteria have invaded the tissue, and bacterial components initiate the innate immune response. Chemokines and cytokines have promoted leukocyte and endothelial activation. The various stages with MMP-7, -8 and -9 involvement are indicated with letters A-H. A) Activated neutrophils attach to the endothelial surface and release granular contents including MMPs. They migrate through the capillary wall towards a chemokine gradient. Basement membrane and ECM components are digested by proteinases, leading to increased permeability and tissue damage. B) MMPs released by neutrophils, macrophages and resident cells digest the ECM. Loss of cell-ECM contact causes anoikis-like cell death. ECM fragments also work as alarmins. Fragmented ECM facilitates cell invasion, but possibly also bacterial spread. C) MMPs activate IL-8 to a more potent chemokine and process other chemokines. D) They activate IL-1 β , but also degrade the mature cytokine. Cytokines upregulate MMP expression in cells. By degrading the ECM structure, MMPs liberate resting cytokines and growth factors harboured in the matrix. E) MMPs shed membrane-bound TNF- α to a soluble biologically active cytokine, which upregulates also MMP expression and inhibits neutrophil apoptosis. F) MMPs may directly activate nuclear factor Kappa B promoting the expression of pro-inflammatory cytokines and MMPs. G) MMP-7 sheds Fas ligand, which may then attach to its receptor and promote epithelial cell apoptosis. H) MMPs are released into the circulation. In sepsis, neutrophils degranulate in an uncontrolled manner. This may promote circulatory changes and changes in coagulation and cause tissue damage.

Experimental studies investigating MMP-8 and -9 levels in different sepsis models are summarized in Table 2. In general, sepsis is associated with increased local and systemic MMP levels, and the synthesis of the enzymes is upregulated. In several studies, mortality is decreased (Maitra et al. 2003, Hu et al. 2005, Steinberg et al. 2003, Vandenbroucke et al. 2012, Solan et al. 2012), and organ damage can be prevented or alleviated by using an MMP inhibitor (Maitra et al. 2003, Steinberg et al. 2003, Steinberg et al. 2005). Because the available MMP inhibitors are non-selective regarding the inhibited MMP and some are also TACE inhibitors, it is not possible to differentiate the roles of individual MMPs based on these studies. Studies on knock-out (genetically MMP-8 or -9-deficient) mice may shed light on this question. MMP-8-deficient mice show better survival and reduced organ

damage in sepsis models compared with their WT counterparts (Van Lint et al. 2005, Vandenbroucke et al. 2012, Solan et al. 2012). They express less chemokines (Van Lint et al. 2005) and pro-inflammatory cytokines (Solan 2012 et al., Vandenbroucke et al. 2012), and more anti-inflammatory IL-10 than WT mice (Solan et al. 2012). Although the chemokine levels were lower and the animals had less neutrophil infiltration in tissues, no differences were present in the clearance of bacteria (Solan et al. 2012). These results suggest a deleterious role for MMP-8 in sepsis. The results from MMP-9 knock-out models are controversial. Dubois et al. (2002) found better survival in MMP-9-deficient mice, whereas in another study lack of MMP-9 was associated with more severe organ damage, decreased neutrophil influx and impaired clearance of pathogens (Renckers et al. 2006). It should be noted that the sepsis models were different; the model in the latter study resembled human sepsis more closely.

In healthy volunteers, infusion of lipopolysaccharide causes a rapid upregulation of proMMP-9 in the circulation, peaking as early as 1.5-3 hours (Pugin et al. 1999^a, Albert et al. 2003). This initial upregulation is probably due to immediate release from activated neutrophils because in other cell types MMP-9 is released slower in response to LPS stimulation *in vitro* and is probably due to increased synthesis (Pugin et al. 1999^a). In a small study of septic patients, plasma MMP-9 was increased during the first day of sepsis, subsequently diminishing (Mühl et al. 2011). TIMP-1 was elevated throughout the study period, days 1-5 of severe sepsis in the same study (Mühl et al. 2011).

Table 2. Studies on MMP-8 and MMP-9 in different experimental sepsis models.

Author, year	Animals	Sepsis model	Measures	Inhibitor	Outcome	Other	Other
Descriptive studies							
Paemen et al. 1997	Baboons	<i>Escherichia coli</i> i.v.	serum MMP-9	-	-	Upregulation of MMP-9 2h after injection	MMP-9 elevated faster than MCP-2 chemokine
Cuenca et al. 2006	Rats	LPS	MMP-9 expression in cardiac myocytes and resident non-myocytic cells			MMP-9 expression increased after LPS in cardiomyocytes	Increased infiltration of inflammatory cells. NOS-2 inhibitor and COX-2 inhibition decrease MMP-9 expression
Castellheim et al. 2008	Pigs	<i>E.coli</i> i.v.	MMP-9 level and activity higher in test animals	-			TNF- α , IL-1 β , IL-6, IL-8 and IL-10 increased
Maitra et al. 2010	Rats	CLP	Liver expression of MMP-9 and TIMP-1 protein and gene expression	-	-	MMP-9/TIMP-1 ratio lowered in septic animals	
Studies using an MMP inhibitor							
Maitra et al. 2003	Rats	CLP	Survival, plasma and tissue MMP-9	CMT-3, hydroxamate	24 h mortality reduced by inhibitors	Reduction of MMP-9 elevation in plasma and liver by inhibitor	Reduction of hepatic transaminases by inhibitor. Reduction of nitrate in plasma
Steinberg et al 2003	Rats	CLP	Survival, degree of lung damage by histology, wet-to-dry ratio	COL-3 (CMT) (inhibits MMP and NE)	Improved survival by inhibitor, better with repeated doses vs. single dose	Lung damage diminished, less lung water and alveolar wall thickening with inhibitor	Similar neutrophil accumulation. Follow-up 7 days
Hu et al. 2005	Mice	LPS	Survival after different doses LPS	Regasepin 1	Improved survival with inhibitor i.p. or i.v.		Regasepin1 inhibits MMP-8, MMP-9 and TACE <i>in vitro</i>
Steinberg et al. 2005	Pigs	Mesenterial ischaemia/ reperfusion and faecal blood clot	Development of ARDS, BAL MMP-2 and MMP-9, elastase, serum /BALF cytokines, histology	COL-3		Inhibitor prevented lung injury, shock, platelet decrease and lactataemia. Increased urine output	Lower IL 6, IL-8, IL-10 and NE by inhibitor. No difference in plasma MMP-9, NE, IL-8, IL-10. Pulmonary histology better and less oedema by inhibitor

MMPknock out models							
MMP-8 -/-							
Van Lint et al. 2005	MMP-8 -/- mice	TNF- α +galactosamine i.p.	Survival, histological changes, markers of liver damage and apoptosis		Better survival in MMP-8 -/-	Lower transaminases, diminished neutrophil infiltration	Diminished LIX chemokine release
Vandenbroucke et al. 2012	MMP-8 -/- mice	LPS renal ischaemia/ reperfusion, CLP		-BB-94 (LPS +CLP model) -MMP-8-specific inhibitor	100% survival in knock-outs and WT+inhibitor in LPS and CLP Better survival in knock-outs in renal I/R	CNS barrier leakage demised in knock-outs and inhibitor (LPS, renal I/R) No difference in vascular integrity between MMP-/- and WT Bone marrow tx indicated that MMP-8 plays a role in later stages of endotoxaemia	Lower serum IL-1 β , IFN γ , IL-6 in knock-outs after LPS Longer clotting times, more severe lung and intestinal injury, elevated transaminases in WT than knock-outs after LPS Glucocorticoid receptor downregulated in only MMP-8 +/- brain
Solan et al. 2012	MMP-8 -/- mice	CLP		Hydroxamate	Better survival in knock-outs Better survival with inhibitor	No difference in bacterial clearance	Less lung MPO in MMP-8 -/- mice. Lower IL-6 and IL-1 β , higher IL-10 in MMP-8 -/- mice. Inhibitor lowers IL-4, IL-1 β , MIP-1 α , and TNF- α
MMP-9 -/-							
Dubois et al. 2002	MMP-9 -/- mice	LPS i.v.	Survival	-	Better survival in MMP-9 -/- mice		Similar upregulation of cytokines, MMP-8 and TIMP-1
Renckers et al. 2006	MMP-9 -/- mice	<i>E. coli</i> i.p.			More severe organ damage in MMP-9 -/- mice (liver, lungs) Enhanced bacterial growth in MMP-9 -/- mice though unchanged phagocytosis. Reduced PMN influx in MMP-9 -/-, higher chemokines and pro-inflammatory cytokines	MMP-9 expression increased in plasma, lung, and liver WT mice	Cellular source vascular endothelium and leukocytes

MMP= matrix metalloproteinase; LPS= lipopolysaccharide; MCP-2= monocyte chemoattractant protein-2; NOS-2= nitric oxide synthase-2; COX-2= cyclooxygenase-2; TNF- α = tumour necrosis factor- α ; IFN- γ = interferon- γ ; IL= interleukin; MIP-1 α = macrophage inflammatory protein-1 α ; CLP= caecal ligation and puncture; TIMP= tissue inhibitor of metalloproteinases; CMT= chemically modified tetracycline; NE= neutrophil elastase; MPO= myeloperoxidase; TACE= TNF-alpha converting enzyme; ARDS= acute respiratory distress syndrome; BAL= broncho-alveolar lavage; WT= wild-type; CNS= central nervous system

Table 3. Clinical studies on MMP-8, MMP-9 and TIMP-1 in association with sepsis.

Author, year	Design	Patients and controls	Samples	MMP-8	MMP-9	TIMP-1	Other	Outcome measure	Comment
Nakamura et al. 1998	Prospective observational	20 septic shock patients 25 healthy volunteers	Plasma Timing regarding onset of sepsis not stated. Samples before and after haemoperfusion. Monocyte MMP-9 mRNA	-	Higher in patients than controls. Higher in non-survivors. Lower after haemoperfusion.	-	Polymyxin B haemoperfusion lowered endotoxin and MMP-9	Mortality	Monocyte MMP-9 mRNA higher in non-survivors
Yassen et al. 2001	Prospective observational	10 patients with severe sepsis 12 non-septic critically ill patients and 8 healthy controls	Plasma on study entry, 24 and 48 hours from 1 st sample. Inclusion within 12 hours of fulfilling severe sepsis criteria	-	Higher in patients than healthy controls. No difference between septic and non-septic patients	-			
Hoffmann et al. 2006	Prospective observational	37 severe sepsis patients (86% lung infection) 37 healthy volunteers	Plasma within 24 hours of diagnosis	-	Higher in patients. No difference between survivors and non-survivors	Higher in patients. Higher in non-survivors than survivors. AUC 0.78 (p<0.01), RR 4.5; 95%CI 1.14-17.6 at TIMP >3200 ng/ml	MMP-2, TIMP-2, IL-6	Overall mortality (28-day) 32.4%	1 st study to describe mortality association. Relatively low APACHE II.
Lorente et al. 2009	Prospective multi-centre observational	192 severe sepsis patient 50 age- and sex-matched healthy controls	Serum at time of diagnosis	-	No difference between patients and controls. Lower in non-survivors than survivors.	Higher in patients than controls. Higher in non-survivors than survivors. AUC=0.68, RR 1.8 at TIMP-1>531 ng/ml	MMP-10, TNF- α , IL-10. IL-10 higher in nonsurvivors	ICU mortality	MMP-9 and TIMP-1 correlated positively with SOFA, lactate, markers of coagulopathy

Gäddnäs et al. 2010	Prospective observational	44 adults with severe sepsis 15 healthy volunteers	Serum on days 1 (within 48 hours of organ dysfunction), 4, 6, 8, 10, 3 months and 6 months. Skin blister samples	Higher in patients d10. No difference in serum levels between survivors and non-survivors	Lower in patients d10. No difference in serum levels between survivors and non-survivors. Higher in skin blisters in MOF vs. MODS	-	MMP-2 higher in patients. Higher skin blister MMP-2 in non-survivors and MOF vs. MODS	ICU, hospital and d-30	
Yazdan-Ashoori et al. 2011	Prospective observational	20 severe sepsis patient 15 healthy controls	Plasma within 24 hours of severe sepsis criteria, daily 7 days, then once weekly	Elevated 35-fold vs. controls	Elevated 4,3-fold. In 50% of patients active form present in zymography on day 1. Greatest concentration on day 1	2.1-fold increase	rhAPC was not associated with MMP-9 levels	MMP-7 and -9 correlated negatively with MOD scores	
Solan et al. 2012	Retrospective observational	32 children with sepsis, 98 with septic shock. Another 180 children with septic shock 32 healthy controls	Plasma within 24 hours of dg and 48 hours thereafter	Whole blood derived mRNA; MMP-8 activity by fluorimetry. MMP-8 mRNA and enzyme activity higher in patients than controls. Higher in septic shock compared with sepsis. Higher MMP-8 mRNA in non-survivors				28-day mortality	MMP-8 mRNA was associated with severity of organ failure

MMP= matrix metalloproteinase; mRNA= messenger RNA; TIMP-1= tissue inhibitor of metalloproteinases; IL= interleukin; APACHE II= acute physiology and chronic health evaluation II; TNF- α = tumour necrosis factor- α ; AUC= area under the curve; RR= relative risk; ICU= intensive care unit; SOFA= sequential organ failure assessment; MOF= multiple organ failure; MODS= multiple organ dysfunction syndrome; rhAPC= recombinant human activated protein C

The clinical studies investigating MMP-8 or -9 in septic patients are few and small. The studies are summarized in Table 3. Three studies report elevated MMP-9 in patients relative to controls. One study found no difference, and in one study MMP levels were lower in patients. In one study, no difference existed between septic and non-septic critically ill patients. Nakamura et al. (1998) reported higher levels of MMP-9 in non-survivors; whereas in two studies there were no differences in MMP-9 levels and in one study MMP-9 levels were higher in survivors (Lorente et al. 2009). Elevated MMP-8 expression was associated with decreased survival in a retrospective study (Solan et al. 2012), while no difference was found in another small study (Gäddnäs et al. 2010).

Interestingly, TIMP-1 levels were associated with increased mortality in two studies (Hoffman et al. 2006, Lorente et al. 2009).

2.2.3. ORGAN DYSFUNCTION

Increased endothelial and epithelial basement membrane permeability, neutrophil accumulation and damage to tissue architecture are all features of sepsis-associated organ dysfunction (Fry 2012). MMPs have been suggested to be involved in these processes because of their ability to process ECM components and to tune and amplify immune reactions. In a mouse model of multiple organ dysfunction, initiated by zymosan-induced peritonitis, expression of MMP-9 mRNA was detected in several distant organs and active enzyme was detectable especially in the spleen and the liver (Volman et al. 2004). This upregulation was visible at 5-12 days after the initial insult, but the authors did not correlate the findings with clinical parameters of organ dysfunction. A similar enhanced MMP-9 expression and corresponding upregulation of MMP-9 protein in several distant organs was seen in LPS-induced endotoxaemia in mice. Concomitant upregulation of the TIMP-1 gene was also noted (Pagenstecher et al. 2000). In a moderate-sized multi-centre study on septic patients, Lorente et al. (2009) found that MMP-9 and TIMP-1 correlated positively with SOFA, lactate and markers of coagulopathy. Another study reported increased MMP-9 levels in skin blister fluid in patients with sepsis-associated MODS (Gäddnäs et al. 2010). In addition, MMP-8 mRNA was associated with severity of organ failure in a recent small retrospective study on septic children (Solan et al. 2012). MMP-8 and -9 are reviewed below in the context of sepsis-induced organ dysfunction.

2.2.3.1. Kidney

In a study by Pagenstecher et al (2000) the localisation of increased gelatinolytic activity in the kidney was seen predominantly in the walls of small vessels. The role of MMP-8 and -9 in human acute kidney injury has been poorly investigated. Recently, however, serum MMP-8 was found to be a sensitive but non-specific biomarker in predicting sustained fluid resuscitation-resistant septic shock-associated AKI in paediatric patients (Basu et al. 2011).

2.2.3.2. Coagulation

No clinical studies investigating the role of MMP-8 or -9 in sepsis-associated disseminated intravascular coagulation exist. However, MMP-8 and -9 have intriguing functions in different stages of coagulation and fibrinolysis. Especially MMP-9 seems to act both in favour and against clot formation. Neutrophils degranulate and release MMP-8, MMP-9 and TIMP-2 in response to stimulation with recombinant tissue plasminogen activator (Cuadrado et al. 2008). MMP-8 cleaves tissue factor pathway inhibitor (TFPI), the primary inhibitor of the tissue factor pathway of coagulation, *in vitro*, leading to a diminished inhibitory action of TFPI on factor Xa (Cunningham et al. 2002). MMP-7 and -9 are also capable of cleaving TFPI (Belaouajij et al. 2000). Human platelets secrete MMP-9 in response to thrombin stimulation, and MMP-9 inhibits thrombin-induced platelet-aggregation (Fernandez-Patron et al. 1999). The synthesis of MMP-9 is upregulated by tissue plasminogen activator (Hu et al. 2006). MMP-9 binds to fibrin and is activated by a plasmin-mediated pathway (Baramova et al. 1997, Makowski et al. 1998). MMP-7 can solubilize cross-linked fibrin (Bini et al. 1996). MMP-8 is able to cleave fibrinogen into fragments, thus impairing its coagulation potential (Hiller et al. 2000). In a recent study on MMP-8-deficient mice, WT mice had significantly longer coagulation times than knock-out mice after LPS stimulation (Vandenbroucke et al. 2012).

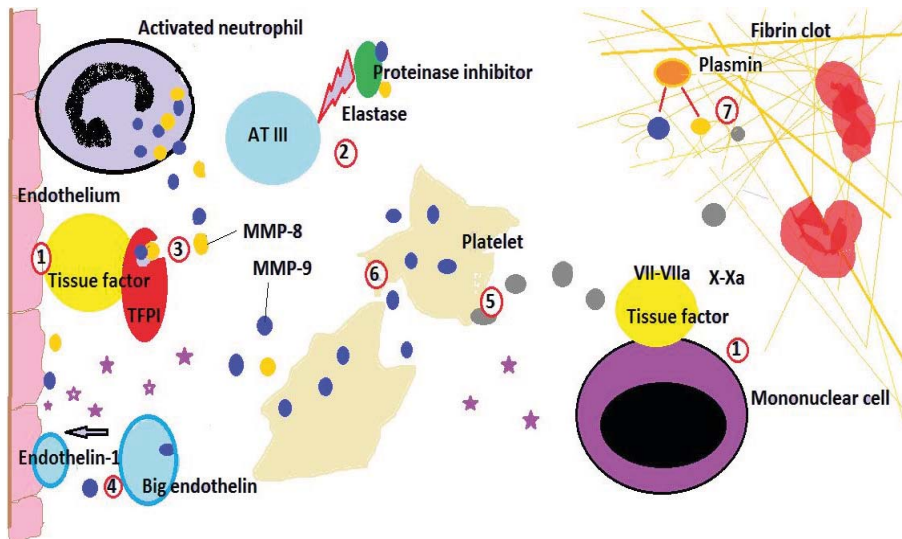


Figure 3. Suggested roles for MMPs in coagulation and fibrinolysis. 1. Various cells express tissue factor, which upon tissue damage activates the extrinsic coagulation pathway, leading to the activation of factors VII and X. This is inhibited by tissue factor pathway inhibitor (TFPI). Uninhibited, this leads to activation of prothrombin to thrombin, which converts fibrinogen to fibrin. 2. AT III is a major physiological anticoagulant, that may be inactivated by elastase. MMP-8 and -9 inactivate proteinase inhibitor, which is a potent inhibitor of elastase. This may lead to potentiation of AT III degradation by elastase. 3. Both MMP-8 and MMP-9 inactivate TFPI by cleavage and may thus contribute to activation of the extrinsic pathway. 4. MMP-9 cleaves big endothelin to vasoactive endothelin, thus potentially contributing to local vasoconstriction. 5&6. Thrombin stimulates platelets to release MMP-9, which acts on platelet cell membrane to decrease aggregation. 7. Plasmin activates MMP-8 and MMP-9. They may bind to fibrin to inactivate it by cleavage, thus decreasing clot formation.

2.2.3.3. Brain

Septic encephalopathy, i.e delirium, is usually an early feature of sepsis-associated organ dysfunction. The pathophysiological mechanisms are not well known, but one of the suggested mechanisms involves increased permeability of the blood-brain barrier (BBB) and a subsequent influx of pro-inflammatory mediators (reviewed in Ebersodt et al. 2007). MMPs may well play a role in BBB disruption, because MMP-9 cleaves BBB effectively in experimental infection and also in ischaemia/reperfusion-associated SIRS (Paul et al. 1998, Rosenberg et al. 1996). Even distant organ damage may trigger this permeability change, at least experimentally. Namely, peripheral thermal injury is associated with increased MMP-9 mRNA in brain tissue, coinciding with increased BBB permeability (Swann et al. 2007). In addition to the BBB, MMPs may alter the permeability of other important barriers. Recently, the important role of MMP-8 in disrupting the blood-cerebrospinal fluid barrier (blood-CSF barrier) in sepsis and renal ischaemia-reperfusion-induced SIRS was demonstrated by Vandenbroucke et al. (2012). Blood-CSF barrier, formed by the chorioid plexus, was protected from disruption in MMP-8 *-/-* mice and by using an MMP inhibitor. An intact chorioid plexus was associated with a decreased inflammatory response

(Vandenbroucke et al. 2012). The blood-CNS barrier has been described as the immune monitor of the brain, which in turn exerts immunomodulatory functions on the whole body. By affecting the patency of this barrier, MMP-8 may be involved in the regulation of systemic inflammation (Vandenbroucke et al. 2012, Ebersoldt et al. 2007). However, MMPs may also exert important physiological functions in neuronal maintenance and reparative processes. In experimental studies, MMP-9 seems to affect neuronal plasticity and promote memory and learning (Nagy et al. 2006, Meighan et al. 2006). Interestingly, a recent study found that in critically ill patients lower plasma MMP-9 levels were associated with increased risk for delirium, but the mechanism remains unclear (Girard et al. 2012).

2.2.3.4. Liver

A potential role of MMP-8 in hepatic tissue damage was suggested by Van Lint et al. (2005), who found that mice genetically deficient in MMP-8 were resistant to TNF- α -induced lethal hepatitis, probably because of impaired neutrophil influx to the liver. This was thought to be due to lack of chemokine production in knock-out mice. On the other hand, lack of MMP-9 seemed to lead to more severe liver damage in an experimental mouse peritonitis model (Renckers et al. 2006).

2.2.3.5. Circulation

In endotoxaemic rats, vascular hyporeactivity to vasoconstrictors can be attenuated by administering doxycycline, an MMP inhibitor (Lalu et al. 2006). This seems to be independent or at least a downstream mechanism of the iNOS (inducible nitric oxide synthase)-mediated pathway (Cena et al. 2010). In septic rat myocardium, cardiomyocytes express increased amounts of MMP-9 (Cuenca et al. 2006). It is well established that in the non-septic failing heart ECM remodelling takes place and MMPs are involved in these processes (reviewed by Tsuruda 2004). In a recent experimental model of acute pulmonary embolism an upregulation of MMP-9 was seen after embolism. By using doxycycline this upregulation and concomitant increase in pulmonary vascular resistance index and mean pulmonary artery pressure could be diminished (Fortuna et al. 2007).

2.3. MMP-8, MMP-9 AND TIMP-1 IN ACUTE LUNG INJURY

MMP-8 and -9 participate in the pathophysiology of various acute and chronic lung diseases such as MMP-9 in interstitial pneumonias (Suga et al. 2000), tuberculosis

(Chang et al. 1996), MMP-8 in bronchiectasis (Sepper et al. 1995) and MMP-8 and -9 in cystic fibrosis (Ratjen et al. 2002), chronic obstructive pulmonary disease (COPD) (Segura-Valdez et al. 2000) and asthma (Vignola et al. 1998, Prikk et al. 2002). Most of these conditions are associated with increased neutrophil infiltration in tissue. In acute infection, such as community-acquired pneumonia, MMP-9 activity and concentration in plasma are elevated (Yang et al. 2005). Similar results of elevated MMP-8 and -9 in mini-BAL (broncho-alveolar lavage) (Hartog et al. 2003, El-Solh et al. 2010) and plasma (Hartog et al. 2003) have been reported in patients with hospital-acquired pneumonia. In these patients, an association of MMPs with clinical severity of the disease was found (Hartog et al. 2003, El-Solh et al. 2010). Infection by highly pathogenic strains of *Pseudomonas aeruginosa* was associated with higher MMP-8 and -9 levels, and a high MMP-9/TIMP-1 ratio was associated with increased alveolo-capillary leakage and mortality (El-Solh et al. 2010). Elevated MMP-8 and -9 in BAL fluids of suspected ventilator-associated pneumonia (VAP) may differentiate true VAP from non-VAP (Wilkinson et al. 2012).

2.3.1. ALI/ARDS DEFINITION AND PATHOPHYSIOLOGY

Acute respiratory failure (ARF) is a common reason for intensive care admissions (Vincent et al. 2002, Linko et al. 2009), and especially its most severe forms, acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), bear high mortality (Vincent et al. 2002, Linko et al. 2009). While ARF lacks a generally accepted definition, ARDS has traditionally been defined by the American-European Consensus Conference criteria which include acute onset of hypoxaemia (ratio of partial pressure of arterial oxygen to fraction of inspired oxygen $[PaO_2/FiO_2] \leq 200$ mmHg for ARDS and ≤ 300 mmHg for ALI), with bilateral infiltrates on chest X-ray, in the absence of left atrial hypertension (Bernard et al. 1994). A new definition of ARDS – the Berlin definition – was recently published and includes three different severity categories of ARDS. It recognizes certain underlying diseases as risk factors for ARDS, but does not include inflammatory parameters in the definition (ARDS Definition Task Force 2012). However, although minor differences are present in the pattern, depending on whether the original insult is direct or indirect, inflammation is a pathophysiologically central feature in the acute phase of lung injury (Ware et al. 2000). Accumulation of neutrophils and platelets, activation of coagulation pathways and altered permeability in the endothelial wall and epithelial structures are typical findings (Matthay et al. 2012). Furthermore, activation of innate immune response *via* Toll-like receptor activation plays an essential role in ALI (Matthay et al. 2012).

2.3.2 THE ECM IN THE LUNG

The ECM in the lung consists of type I collagen, which is responsible for the tensile strength of the lung tissue and is highly resistant to proteolytic enzymes. Elastin provides distensibility of tissue. Endothelial and epithelial basement membranes contain type IV collagen, and the alveolar wall mainly consists of type III collagen (Elkington et al. 2005). MMP-8 and MMP-9 together can cleave all of these ECM constituents, and given their role in several stages of acute inflammation, it is tempting to believe that they may participate in ALI pathogenesis. In experimental studies, MMPs are frequently detectable in early stages of inflammation in various models of lung injury, summarized in Table 4. Studies using different models of injury and animals treated with MMP inhibitors show that by using inhibitors neutrophil accumulation, upregulation of pro-inflammatory mediators, histological tissue damage and permeability changes can almost always be attenuated (Table 5). However, the presently available inhibitors are not specific to MMP-8 or MMP-9, and therefore, their independent roles can not be fully elucidated based on these studies. Studies using genetically MMP-8 or MMP-9-deficient mice may clarify their independent roles and are shown in Table 6.

2.3.3 MMP-8 AND ACUTE LUNG INJURY

MMP-8 is detectable in BAL of healthy volunteers (O' Kane et al. 2009). Only a few clinical studies have assessed MMP-8 levels in ALI, and no studies report systemic MMP-8 levels. In ALI MMP-8 seems to be elevated in BAL or tracheal aspirates.

The source of MMP-8 in lung injury seems to be predominantly neutrophils. MMP-8 in tracheal aspirates of paediatric ARDS correlates strongly with other neutrophil proteinases such as human neutrophil elastase and myeloperoxidase (Kong et al. 2011). However, different cell types may be responsible for the MMP-8 production depending on the model used. In one experimental study, the presence of a lower molecular weight form consistent with mesenchymal MMP-8 was also detected (Cederqvist et al. 2006), and lung fibroblasts were identified as producing MMP-8 in a study using a bleomycin-induced lung inflammation model (García-Pieto et al. 2010). Study results using MMP-8-deficient animals are controversial. MMP-8 knock-out mice seem to have more neutrophil accumulation, permeability changes and capillary injury than WT mice (see Table 6). Neutrophil apoptosis is decreased in knock-out animals and they show higher IL-10, MIP-1 α and alarmin levels. In one study using three different mechanisms of injury, MMP-8^{-/-} mice had higher mortality in all groups (Quintero et al. 2010), whereas another study found no difference in survival, although the initial lung injury was more severe in the MMP-8^{-/-} mice (González-López 2012). In a bleomycin model of lung injury, knock-outs had an increased and prolonged inflammatory infiltrate but less lung fibrosis

Table 4. Experimental studies examining MMP levels in lung injury.

Author, year	Animals	Model of injury	Measures	Results	Other
Gibbs et al. 1999	Rats	Tracheally instilled IgA immune complexes, LPS	BAL MMP-9	MMP-9 elevated in BAL after IgA and LPS	Injury was partly inhibited by TIMP-2
Gushima et al. 2001	Pigs	Hyperoxia	BAL and tissue MMP-2 and -9, correlation with oxygenation, number of neutrophils and wet-to-dry ratio	MMP-2 and -9 increase after 72hours, MMP-9 localized in macrophages, neutrophils and alveolar lining epithelium	MMP-9 correlates well with histological scores of lung injury
Eichler et al. 2003	Pigs	Cardiopulmonary bypass (hypothermic)	BAL MMP-2 and -9, AaDO ₂ , PCWP, lung tissue water content	MMP-2 and MMP-9 increased after CPB, MMPs correlated with AaDO ₂	
Hirsh et al. 2004	Mice	Neutropenic sepsis (cyclophosphamide+CLP)	Tissue homogenate MMP-2 and -9	Elevated MMP-2 and 9 after CLP	Cyclophosphamide induced MMP-9 activity
Cederqvist et al. 2006	Rats	Hyperoxia	BAL and tissue MMP-2,-8,-9, trypsin	MMP-8 and MMP-9 increased after 48 hours	
Castellheim et al. 2008	Pigs	Live E.Coli bronchial instillation	Plasma MMP-9	Plasma MMP-9 elevated, earlier than in i.v. infusion of <i>Escherichia coli</i>	

IgA= immunoglobulin A; LPS= lipopolysaccharide; MMP= matrix metalloproteinase; BAL= broncho-alveolar lavage; TIMP= tissue inhibitor of metalloproteinases; AaDO₂= alveolar-arterial oxygen difference; PCWP= pulmonary capillary wedge pressure; CBP= cardiopulmonary bypass; CLP= caecal ligation and puncture

in the later stages (García-Prieto et al. 2010). Interestingly, in at least one study MMP-9 levels were higher in the lung tissue of MMP-8-deficient mice, suggesting that loss of one enzyme may be compensated by another (García-Prieto et al. 2010).

In humans, at least locally in the lung, MMP levels tend to be elevated in inflammatory states. In healthy volunteers, inhalation of bacterial lipopolysaccharide resulted in elevated MMP-7, -8 and -9 in BAL fluid (Shyamsundar et al. 2009). In a study of 28 patients with ALI/ARDS, elevated levels of MMP-8 were detected in BAL fluids of almost all patients (Fligiel et al. 2006). Similarly, O' Kane et al (2009) reported increased MMP-8 levels in ARDS patients, with unchanged concentrations between days 0 and 4 of the disease. High MMP-8 levels in tracheal aspirate samples were present at 24 (Kong et al. 2009) and 48 hours (Kong et al. 2011) after onset of ARDS in paediatric patients, and MMP-8 levels decreased markedly between days 2 and 6 of disease (Kong et al. 2011).

Although MMP-8 levels are elevated in BAL fluids of ALI/ARDS, it is far from clear whether this is harmful, beneficial or of no relevance. No correlation of MMP-8 was found with any outcome measures, disease severity or oxygenation disorder (Fligiel et al. 2006). On the contrary, high tracheal aspirate MMP-8 in paediatric ARDS patients predicted need for longer mechanical ventilation independently (Kong et al. 2011).

Table 5. MMP inhibition in acute lung injury models.

Author, year	Animal	Lung injury model	Inhibitor	Measures	Inhibitor effect	Other	Outcome (mortality)
Kang et al. 2001	Rats	LPS intra-tracheally	Genistein (protein tyrosine kinase inhibitor)	NF-kappaB activation, BAL protein and LD, CINC, MMP-9 and neutrophil recruitment	Reduced NF-kappaB activation; BAL protein and LD, CINC, MMP-9 and neutrophil recruitment		
Carney et al. 2001	Pigs	LPS intravenously	COL-3 (neutrophil MMP and NE inhibitor)	BAL MMP-2 and -9, tissue neutrophils, lung wet/dry ratio	Reduced MMPs, neutrophil count and wet/dry ratio by inhibitor	High MMPs, neutrophil sequestration and wet/dry ratio in lung after LPS	
Coimbra et al. 2006	Rats	LPS intravenously	Pentoxifylline	BAL IL-8, MMP-2, -9, plasma MMP-9, histology, lung ICAM-1, MPO, NF-kappaB	Reduced IL-8, BAL MMP-2, plasma MMP-9, histological lung damage, MPO, NF-kappaB, ICAM-1		
Carney et al. 1999	Pigs	CPB (hypothermic)+LPS	CMT-3	PaO2 and shunt measurements, gelatinase and elastase activity	Changes prevented reduced elastase and gelatinase activity		
Wang et al. 2012	Rats	CPB (normothermic)	Doxycycline p.o.	BAL MMP-9, TIMP-1, tissue MMP-9-mRNA, TIMP-1 mRNA	MMP-9 decreased and TIMP-1 mRNA increased. Attenuated lung injury.		
Foda et al. 2001	Rats	VILI	Prinomastat	In situ hybridization detection and BAL MMP-2 and -9, MTI-MMP and EMMPRIN mRNA	Lung injury, gelatinase and TNF α levels attenuated	Gelatinases upregulated by high-volume ventilation.	
Kim et al. 2006	Rats	VILI	CMT-3	MMP-9 activity, immunohistochemistry, wet/dry ratio, ALI score, neutrophil infiltration	Decreased MMP-9 activity and immunoreactivity, lung injury measures and neutrophils.		
Doroszko et al. 2010	Rats	VILI	Doxycycline p.o.	Lung tissue proteomics, respiratory mechanics and gas exchange	Better respiratory mechanics and gas exchange and lower MMP-9 activity		
Keck et al. 2002	Rats	Pancreatitis-associated lung injury	Batimastat	Lung homogenates, PMN cultures MMP-2 and -9 excretion, PMN transmigration	Reduced PMN transmigration by inhibitor	MMP-9 highly expressed in AP. Trypsin, IL-1 β , TNF- α stimulated PMN release of MMP-9 in vitro	
Muhs et al. 2003	Rats	Pancreatitis-associated lung injury	Batimastat	Histology, serum MMP-activity, vascular permeability	MMP activity, histological lung damage and vascular permeability attenuated		100% survival by inhibitor versus 50%

2. REVIEW OF THE LITERATURE

Author, year	Animal	Lung injury model	Inhibitor	Measures	Inhibitor effect	Other	Outcome (mortality)
Richter et al. 2009	Rats	Pancreatitis-associated lung injury	Doxycycline	Histology, MMP-9 activity, neutrophil accumulation	Decreased MMP-9 activity and neutrophil accumulation. Decreased histological injury	TNF α -induced PMN migration inhibited by doxycycline in vitro	
Mulligan et al. 1993	Rats	Intrapulmonary immunocomplex-mediated acute alveolitis	Recombinant intratracheal TIMP-2	Permeability, haemorrhage	Reduced damage		
Gipson et al. 1999	Rats	Immune complexes	Antibodies for TIMP-2 and SLPI	BAL cell and protein content	More severe lung damage by inhibitors		
Deree et al. 2007	Rats	Haemorrhagic shock	Hypertonic saline and pentoxifylline versus Ringer's lactate in resuscitation	BAL CINC and MMP-2 and -9	CINC levels and lung MMP-2 and -9 expression reduced by saline-pentoxifylline		

LPS=lipopolysaccharide; NF-kappaB= nuclear factor kappaB; BAL= broncho-alveolar lavage; LD= lactate dehydrogenase; CINC= cytokine-induced neutrophil chemoattractant; MMP= matrix metalloproteinase; NE= neutrophil elastase; BAL= broncho-alveolar lavage; IL= interleukin; ICAM-1=intercellular adhesion molecule-1; MPO= myeloperoxidase; CBP= cardiopulmonary bypass; CMT= chemically modified tetracycline; PaO2= arterial partial oxygen tension; TIMP= tissue inhibitor of metalloproteinases; mRNA= messenger RNA; VILI= ventilator-induced lung injury; MTT-MMP= membrane-type MMP; EMMPRIN= extracellular matrix metalloproteinase inducer; TNF- α = tumour necrosis factor- α ; ALI= acute lung injury; PMN= polymorphonuclear leukocyte; AP= acute pancreatitis; IL= interleukin; SLPI= secretory leukocyte peptidase inhibitor

Table 6. Acute lung injury in MMP-8 and -9 knock-out models.

Author, year	Animal	Model	Measures	Results	Outcome	Other
Owen et al. 2004	MMP-8 -/-	LPS intratracheally	PMN accumulation	2 times greater PMN accumulation in MMP-8 -/- mice		Membrane-bound, TIMP-1-resistant membrane-type MMP-8 demonstrated
Gueders et al. 2005	MMP-8 -/-	Allergen inhalation	BAL, histology, neutrophil apoptosis, measurement of cytokines	Increased IL-4 and neutrophil counts in MMP-8 -/- mice		Decreased neutrophil apoptosis in knock-outs
García-Prieto et al. 2010	MMP-8 -/-	Bleomycin intratracheally	Histology, enzyme activity, MPO	Less lung fibrosis in MMP-8 -/- mice, increased and prolonged inflammatory infiltrate in MMP-8 -/- mice		-MMP-8 elevation in WT, mainly in fibroblasts, TGFβ only in WT, 3-fold increase in IL-10 in knock-outs -IL-10 increase protected from fibrosis (less collagen synthesis) -MMP-9 more elevated in knock-out lung tissue
Quintero et al. 2010	MMP-8 -/-	LPS intratracheally, bleomycin, hyperoxia	BAL, histology, lung elastance, MIP-1α	Greater PMN and macrophage accumulation, more severe lung capillary barrier injury in MMP-8 -/-	Higher mortality in MMP-8 -/- mice in all models of injury	Injury mediated via MIP-1α
González-López et al. 2012	MMP-8 -/-	LPS intraperitoneally	Histology, BAL, proteomic analysis	-More severe lung injury in MMP-8 -/- -More abundant neutrophil influx in MMP-8 -/-	No difference in survival	Accumulation of alarmins S100A8 and S100A9 in lung in MMP-8 -/-
Roscoe et al. 2001	MMP-9 -/- mice	Immune complex-mediated acute alveolitis	BAL MMP-9, morphometry	-High MMP in WT BAL, decreased lung injury in MMP-9 -/ -No difference in neutrophil accumulation		
Chetty et al. 2008	MMP 9 -/- mice	Hyperoxia	Morphometry, MMP-2 and -9, elastin, type I collagen, tropoelastin, compliance	MMP-2 and -9 upregulated in WT mice, WT had less compliant lung with more type I collagen, tropoelastin		Localization of MMP-9 in mesenchyme and alveolar epithelium
Lukkarinen et al. 2009	MMP-9 -/- neonatal mice	IL-1β		IL-1 β/MMP-9 (-/-) mice had more severe alveolar hypoplasia and pulmonary cell death		
Kim et al. 2005	TIMP-1 -/-	Bleomycin	BAL, histology	TIMP-1 mRNA and protein increased after injury in WT mice. Augmented neutrophilia and lung injury in TIMP-1 -/-mice		No difference in chemotaxis or collagen accumulation

MMP= matrix metalloproteinase; LPS= lipopolysaccharide; PMN= polymorphonuclear leukocyte; TIMP= tissue inhibitor of metalloproteinases; BAL= bronchoalveolar lavage; IL= interleukin; MPO= myeloperoxidase; WT= wild-type; TGF-β= transforming growth factor-β; IL= interleukin; MIP-1α = macrophage inflammatory protein-1α

2.3.4. MMP-9 AND ACUTE LUNG INJURY

In ALI the recruited neutrophils and alveolar macrophages are important sources of MMP-9 (Fligiel et al. 2006), and the BAL MMP-9 levels correlate highly with neutrophil count (Ricou et al. 1996, Torii et al. 1997). In BAL of healthy controls, macrophages are the predominant cell type, whereas BAL fluid of ARDS patients is rich in neutrophils and lymphocytes (Torii et al. 1997). MMP-9 is also produced by type II alveolar epithelial cells, distal lung epithelial cells, interstitial fibroblasts and pulmonary endothelial cells (Gibbs et al. 1999, O' Kane et al. 2009). In a recent placebo-controlled study, intravenous salbutamol unexpectedly upregulated MMP-9 production *in vivo*, and the effect was localized to distal lung epithelial cells (O' Kane et al. 2009).

MMP-9 probably has a role in neutrophil recruitment because of its ability to process IL-8, the most potent neutrophil chemokine, into a more active form (Van den Steen et al. 2000). However, in a study on genetically MMP-9-deficient mice with immune complex-mediated alveolitis, no differences emerged between MMP-9-deficient and WT mice in neutrophil accumulation (Roscoe et al. 2001). MMP-9 may also play a role in permeability changes in the acute phase of lung injury, and its levels correlate highly with type IV collagen degradation product 7S (Torii et al. 1997). Contradictory evidence comes from a clinical study in which changes in MMP-9 levels correlated inversely with extravascular lung water content in humans (O' Kane et al. 2009). In knock-out models, MMP-9-deficient mice show decreased lung injury and better lung compliance after injury compared with their WT counterparts (Roscoe et al. 2001, Chetty et al. 2008). On the other hand, MMP-9 may also be important in the healing process and in tissue remodelling; MMP-9 was shown to be involved in the migration of alveolar epithelial cells (Buckley et al. 2001) and to be required for alveolar epithelial wound healing *in vitro* (O' Kane et al. 2009). In an experimental study on rats overexpressing MMP-9, bleomycin-induced lung fibrosis was alleviated, possibly by the ability of MMP-9 to cleave and inactivate IGFBP-3, a promoter of collagen synthesis by fibroblasts (Cabrera et al. 2007). Again, evidence from humans points in the opposite direction, as MMP-9 levels in permeability oedema fluid correlate with procollagen peptide III, a marker of collagen synthesis (Pugin et al. 1999^b).

MMP-9 levels are elevated in ARDS patients compared with healthy controls (Torii et al. 1997) or hydrostatic pulmonary oedema patients (Pugin et al. 1999^b). The levels are elevated early in the course of ARDS (O' Kane et al. 2009) and remain high several weeks in plasma and BAL of those ARDS patients who have a prolonged course of the disease (Ricou et al. 1996).

Fligiel et al. (2006) studied MMP-9 levels in BAL fluids of 28 ALI/ARDS patients and found both latent and active forms of the enzyme in 5- to 80-fold higher concentrations compared with healthy controls, but the MMP-9 levels were

not associated with outcome. BAL fluid MMP-9 levels in ARDS patients were lower than in patients with hospital-acquired pneumonia in a small study by Lanchou et al. (2003). In that study, patients with rapidly resolving ARDS had a higher MMP-9/TIMP-1 ratio on day 4 compared with patients with a longer course of the disease, although the MMP-9 levels were similar. In a study on paediatric ARDS patients, high levels of active MMP-9 in tracheal aspirate samples predicted longer need for mechanical ventilation (Kong et al. 2009) and the levels of active MMP-9 rose from day 2 to 6 of disease (Kong et al. 2011). The systemic MMP-9 levels in ALI are less well known. Ricou et al. (1996) found no difference in plasma MMP-9 concentration between healthy controls and ARDS patients, whereas some of the ARDS patients in another study had increased systemic pro-MMP-9 levels, although the overall pro-inflammatory activity in plasma was low (Pugin et al. 1999^b).

2.3.5. TIMP-1

TIMP-1 is expressed in numerous cell types and after hyperoxia-induced lung injury its mRNA is induced in interstitial fibroblasts, airway cartilage chondrocytes and vascular endothelium (Veness-Meehan et al. 1991). TIMP-1 is present in the BAL fluid (Fliegel et al. 2006, Ricou et al. 1996) and plasma (Ricou et al. 1996) of healthy controls, and markedly elevated levels have been detected in BAL (Fliegel 2006, Ricou 1996, Torii 1997) and plasma (Ricou et al. 1996) of ALI/ARDS patients. In a paediatric study, tracheal aspirate TIMP-1 levels were lower in ARDS patients than in patients intubated for non-pulmonary reasons (Kong et al. 2011). TIMP-1 is reported to be higher in plasma than in BAL (Ricou 1996), which is expected due to the dilution effect of BAL on secretions. TIMP-1 in neither plasma nor BAL seems to be correlated with the severity of lung injury or oxygenation disturbance (Ricou et al. 1996). However, high plasma levels of TIMP-1 were associated with worse outcome in one study (Ricou et al. 1996). In contrast, in another study BAL TIMP-1 was not associated with outcome (Fliegel et al. 2006). The balance of MMP-9 and TIMP-1 may better reflect the pathophysiological state; in two studies, a lower BAL fluid MMP-9/TIMP-1 ratio was associated with more severe course of the disease (Ricou et al. 1996, Lanchou et al. 2003), and the low ratio was mainly due to higher TIMP levels (Ricou et al. 1996). Moreover, in a study of paediatric ARDS patients the BAL MMP-9/TIMP-1 ratio was higher in patients with a shorter (<10 days) course of the disease than those with a longer-lasting disease (Kong et al. 2009). One might speculate that MMP-9 levels are elevated locally and more than the actual level, the balance between the enzyme and its inhibitor might be important in determining the course of the disease towards either normal or exaggerated healing with fibrosis.

On the other hand, TIMP-1 has been suggested to participate in IL-6-mediated lung protection in a hyperoxic lung injury model in mice (Ward et al. 2000). In a study on mice genetically lacking TIMP-1, bleomycin caused more severe lung damage and augmented neutrophil accumulation in TIMP-1-deficient individuals (Kim et al. 2005).

2.3.6. CORRELATIONS WITH OTHER INFLAMMATORY PARAMETERS

Pro-inflammatory activity in permeability oedema fluid correlates highly with IL-8 and pro-MMP-9 and can be almost totally inhibited with anti-TNF- α and an IL-1 receptor antagonist (Pugin et al. 1999^b). In BAL fluids of ARDS patients, MMP-8 and MMP-9 have a strong intercorrelation (Fliegiel et al. 2006). The concentrations of MMP-8 (Fliegiel et al. 2006) and MMP-9 (Fliegiel et al. 2006, Ricou et al. 1996) correlated highly with the neutrophil count in BAL fluid. TIMP-1 correlated strongly with BAL interleukin-6 (Ricou et al. 1996).

2.3.7. MMP-8 AND -9 IN VENTILATOR-INDUCED LUNG INJURY

In experimental studies, MMPs -8 and -9 have been described to participate in early phases of ventilator-induced lung injury (VILI). Whereas ventilation with high pressure rapidly induced MMP-8 in the WT animals, mice genetically lacking MMP-8 had less tissue damage, significantly milder oxygenation disorder and smaller amounts of neutrophil infiltration. This harmful effect seemed to be independent of the collagenolytic effect of MMP-8 and rather due to its immunomodulatory properties, with lower chemokine expression and higher anti-inflammatory cytokines IL-4 and IL-10 in MMP-deficient animals (Albaiceta et al. 2010). Mice genetically lacking MMP-9, by contrast, exhibited more serious lung damage than their WT counterparts, suggesting that in VILI MMP-9 could be protective (Albaiceta et al. 2008).

2.4. MMPS IN ACUTE PANCREATITIS

Acute pancreatitis is an example of a non-infectious condition where systemic inflammation is highly relevant and early capillary permeability disturbances are typical. Therefore, the presence of MMPs would not be surprising. Some experimental evidence of MMP-9 in association with AP exists, but to date no studies on MMP-8 or clinical studies on either MMP have been published.

Experimental acute pancreatitis is characterized histologically by extensive acinar cell necrosis, haemorrhage, extensive neutrophil infiltration in the pancreatic tissue and activation of trypsinogen to active trypsin (Awla et al. 2012). These pathophysiological changes can be alleviated by an MMP inhibitor and they are reduced in MMP-9-deficient mice. Furthermore, MMP-9 seems to be an important mediator in activating trypsinogen in the acinar cells, which is a central pathophysiological feature in acute pancreatitis (Awla et al. 2012). On the other hand, trypsin is able to activate MMP-9 (Sorsa et al. 1997), thus creating a possible positive feedback loop. MMP-9 is elevated and extensively activated in the peritoneal fluid in experimental acute pancreatitis in rats (Muhs et al. 2001), and it may be pathophysiologicaly relevant since by using a broad-spectrum MMP inhibitor, Batimastat, local and distant organ injury associated with experimental pancreatitis can be inhibited (Muhs et al. 2003). Neutrophils are involved in the pancreatitis-associated lung injury (Awla et al. 2012). Elevated MMP-9 in the lung tissue coincides with neutrophil infiltration, which is reduced with an MMP inhibitor (Keck et al. 2002). Furthermore, elevated MMP-9 has a good predictive value for predicting disease severity and pulmonary complications in acute pancreatitis models (Keck et al. 2006).

2.5. MMPS AND CARDIAC ARREST

Another example of systemic inflammation triggered by a non-infectious mechanism is ischaemia-reperfusion-associated inflammatory syndrome. In addition to the immediate hypoxic-ischaemic damage to cells, tissue damage caused by ischaemia-triggered inflammation occurs. The process is further potentiated by reperfusion-induced oxidative and nitrosative stress, leading to the accumulation of reactive oxygen and nitrogen species and activation of the inflammatory cascades. In ischaemia-reperfusion-associated tissue damage, neutrophil granulocytes and their products play a major role, especially in the development of microvascular damage and altered permeability (Granger et al. 1988).

2.5.1. POST-CARDIAC ARREST SYNDROME

Cardiac arrest causes ischaemia and, after a successful resuscitation, reperfusion to all organs of the body. A term describing the entity of pathophysiological changes after successful resuscitation from cardiac arrest has been coined, namely the post-resuscitation disease (Negovsky et al. 1995) or, more recently, post-cardiac arrest syndrome (Nolan et al. 2008). Accumulating evidence suggests that upregulated systemic inflammatory mediators may have a role in the associated phenomena. A rapid upregulation of cytokines and soluble TNF- α receptor II, the magnitude resembling septic states (Adrie et al. 2000), and slightly later adhesion molecules (Gando et al. 2000, Geppert et al. 2000) are seen after the return of spontaneous circulation (Callaway et al. 2008). As a sign of neutrophil activation, neutrophil elastase is rapidly upregulated, and elevation of soluble thrombomodulin marks endothelial damage (Gando et al. 2000). SIRS as a clinical syndrome is a frequent finding in cardiac arrest patients (Geppert et al. 2000).

In addition to brain injury, clinically important signs of inflammatory response, cardiac dysfunction, coagulation abnormalities and adrenal dysfunction have been described (reviewed by Adrie 2004). The degree of cardiac dysfunction was associated with TNF- α levels in a porcine model (Niemann et al. 2004).

2.5.2. MMPS AND NEURONAL DAMAGE

MMPs, particularly MMP-7 and MMP-9, are pathophysiologicaly relevant in several neurological diseases associated with inflammation and disruption of the blood-brain barrier (BBB), such as human immunodeficiency virus-associated dementia (Conant et al. 1999) and multiple sclerosis (Cossins et al. 1997). The BBB consists of laminin, fibronectin, heparan sulphate and type IV collagen (Yurchenco et al. 1990), which are all substrates for MMP-9.

In the brain, endothelial cells, neurons, microglia, astrocytes and oligodendrocytes secrete MMP-9 (Conant et al. 1999, Rivera et al. 2002, Rosenberg et al. 2001), and the secretion can be stimulated by TNF- α (Conant et al. 1999). MMPs may cause tissue injury in the brain in several ways, e.g. degradation of the ECM, disruption of the BBB followed by oedema formation and influx of inflammatory cells, cleavage of cytokines and promoting apoptosis (Lo et al. 2002). For example, injecting TNF- α into the brain causes upregulation of MMP-9 and a simultaneous increase in the permeability of the BBB, which can be inhibited by an MMP inhibitor (Rosenberg et al. 1995). Thus, MMP-9 may act as a final common pathway in permeability changes induced by cytokines (Rosenberg et al. 1995). Activated MMP-9 promotes neuronal apoptosis *in vitro* (Gu et al. 2002). However, MMPs may also exert neuroprotective functions, and they probably play a role in ECM remodelling, revascularization and neuronal growth after injury (Morancho et al. 2010).

2.5.3. MMP-9 IN ISCHAEMIC STROKE

In experimental stroke, microvascular occlusion and accumulation of neutrophils are early features. By 6-12 hours from the insult, neutrophils have invaded the brain parenchyma (Garcia et al. 1994, Akopov et al. 1996), and the abundance of neutrophils is associated with outcome (Akopov et al. 1996). Substantial damage to the microvascular basement membranes with a loss of basement membrane components, including laminin, fibronectin and type IV collagen, is characteristic after cerebral ischaemia and reperfusion (Hamann et al. 1995). MMP-9 participates in the breakdown of the basement membranes during experimental focal ischaemia (Rosenberg et al. 1996). Upregulation of MMP-9 can be seen after experimental focal ischaemia in the neurons, accumulated neutrophils (Rosenberg et al. 2001) and cerebral vessels (Wagner et al. 2003). In patients with acute stroke, serum MMP-9 levels rise early, within 3 hours (Montaner et al. 2003), peak at 24 hours (Montaner et al. 2001^a) and stay elevated for at least 12 days (Horstmann et al. 2003). MMP-9 levels correlate with infarct volume and are associated independently with clinical severity of stroke (Montaner et al. 2001^a). Elevated plasma MMP-9 independently predicts spontaneous (Montaner et al. 2001^b, Castellanos et al. 2003) and t-PA treatment-associated (Montaner et al. 2003) haemorrhagic transformation of cerebral infarct with a relatively good sensitivity and specificity (Castellanos et al. 2003, Montaner et al. 2003). MMP-9 levels are associated with the severity of the haemorrhage (Montaner et al. 2003). Furthermore, patients with a large medial cerebral artery infarct express higher MMP-9 and cellular fibronectin levels if they are to develop a malignant MCA infarct, defined by excessive brain oedema (Serena et al. 2005). In addition to pro-inflammatory actions, at least some MMPs, such as MMP-3 may be important in protecting neurons from delayed death via apoptosis (reviewed by Cunningham 2005). Monocytes from stroke patients express increased levels of MMP-7 mRNA, MMP-9 mRNA and TIMP-1 mRNA (Kouenhowen et al. 2001). In experimental stroke, TIMP-1 levels in the brain tissue increase by 48 hours after ischaemia (Rosenberg et al. 1998). Serum TIMP-1 in patients seems to be unaffected by ischemic stroke (Horstmann et al. 2003).

2.5.4. MMPS AND TIMP-1 IN GLOBAL CEREBRAL ISCHAEMIA

Ischaemic stroke represents focal cerebral ischaemia with damage to the BBB and a fulminant inflammatory response, while cardiac arrest causes transient ischaemia and reperfusion with delayed damage to selective neuronal populations such as hippocampal neurons or caudate nucleus neurons (reviewed in Cunningham 2005). A local upregulation of pro-inflammatory cytokines is seen, but less leukocyte infiltration and BBB damage are present in global ischaemia (Stoll et al. 1998). After

global ischaemia in rodents, active MMP-9 is upregulated in microglia, astrocytes (Rivera et al. 2002) and neurons at such brain regions as hippocampus (Rivera et al. 2002, Lee et al. 2004) and nucleus caudatus, preceding neuronal death (Magnoni et al. 2004). The damage to hippocampal neurons was significantly reduced in MMP-9 knock-out mice or by using a broad-spectrum MMP inhibitor BB-94 (Lee et al. 2004).

TIMP-1 was increased after global cerebral ischaemia (Romanic et al. 1998, Rivera et al. 2002). TIMP-1 protected hippocampal neurons from glutamate excitotoxicity in culture, at least partially independent of its MMP inhibitory actions (Tan et al. 2003).

2.5.5. POSSIBLE THERAPEUTIC MECHANISMS OF MILD HYPOTHERMIA TREATMENT (MTH)

In 2002, two clinical trials showed that mild therapeutic hypothermia improved neurological outcome and survival of comatose out-of-hospital cardiac arrest patients (Bernard et al. 2002, Hypothermia After Cardiac Arrest Study Group 2002). Therapeutic hypothermia is currently a standard treatment for comatose out-of-hospital cardiac arrest patients, particularly those who have ventricular fibrillation as the presenting arrhythmia (Nolan et al. 2003). However, it is not fully clear how hypothermia treatment protects cells from injury after cardiac arrest. Mechanisms such as attenuated inflammatory response, decreased metabolic rate and, thus, decreased oxygen demand, altered neurotransmitter release, moderate anticoagulant effect, and inhibition of vasoconstrictive substances have been proposed (reviewed in Campos 2012). From animal studies on focal ischaemia, hypothermia is known to reduce infarct size and preserve the BBB, with a concomitant reduction in MMP-9 levels and activity in the area of BBB breakdown (Wagner et al. 2003). In a mouse model of focal ischaemia, hypothermia reduced MMP-9 and plasminogen activators in the brain, thus inhibiting the breakdown of type IV collagen in the BBB (Burk et al. 2008). Hypothermia also reduced the ischaemia-induced expression of the apoptosis receptor Fas and decreased the soluble form of the Fas ligand, thus reducing apoptosis. Similar effects were seen by using an MMP inhibitor, and combining the inhibitor and hypothermia produced additive effects (Liu et al. 2008). In a clinical study comparing systemic MMP-9 levels between different treatment groups in stroke patients, MMP-9 levels were lowest in patients treated with MTH (Horstmann et al. 2003). MMP-9 levels correlated positively with body temperature in another study on stroke patients who did not receive hypothermia treatment (Montaner et al. 2001). Considering the results of these studies on focal cerebral ischaemia, the protective mechanisms of hypothermia might include suppression of MMP-9 also in the context of global cerebral ischaemia. Evidence from studies

on hypothermia in other disease entities, such as traumatic brain injury support the hypothesis that hypothermia may be associated with suppressed MMP-9 levels. In experimental traumatic brain injury, post-traumatically elevated MMP-9 in brain tissue was suppressed in the animals treated with mild hypothermia (Truettner et al. 2005).

2.5.6. MTH AND SYSTEMIC INFLAMMATION

Callaway et al. (2008) did not find any difference in the systemic cytokine levels between normothermia- and hypothermia-treated rats, but expression of ICAM-1 adhesion molecules was slightly delayed in the hypothermia group. In a clinical observational study of 71 out-of-hospital cardiac arrest patients, MTH did not affect TNF- α , but the levels of IL-6 increased, though late, in the hypothermia group. However, in that study the MTH group had markedly increased prevalence of bacterial colonization, which may have affected the inflammatory parameters, and the authors did not report time intervals from basic CPR or ROSC. Furthermore, they did not find a difference in survival or neurological outcome between MTH and normothermia groups, in contrast to other studies (Fries et al. 2009). In a model of experimental intestinal ischaemia, hypothermia treatment prevented neutrophil accumulation into the lungs, thus providing indirect evidence that hypothermia treatment might protect organs from ischaemia-reperfusion-induced systemic inflammation (Vinardi et al. 2003).

2.6. PERFORMANCE AND INTERPRETATION OF STATISTICAL ANALYSES OF A BIOMARKER

A biomarker is a “*quantifiable measurement of biological homeostasis that defines what is normal, therefore providing a frame of reference for predicting or detecting what is abnormal*” (Dalton et al. 2006).

For a biomarker to prove useful in clinical practice, it should be shown to differ significantly between healthy and diseased individuals, possess advantageous diagnostic properties, perform better or non-inferiorly relative to existing tests and increase the information available for clinical decision-making. It should also be demonstrated to modify outcome when used in decision-making. Furthermore, the pathophysiological basis should be understood (Ray et al. 2010).

To evaluate the diagnostic or predictive properties of a biomarker, statistical terms, such as sensitivity, specificity, positive and negative predictive values and likelihood ratios, are calculated. These terms are also useful for comparing

performance of a test with others and for determining whether the test gives additional information for clinical decision-making.

When examining the performance of a biomarker in diagnostics or outcome prediction, the sensitivity, specificity and positive and negative predictive values of the marker can be calculated by using a matrix:

Table 7. Matrix for calculating statistical metrics

Test	Disease present	Disease absent
Positive	A	B
Negative	C	D

Modified from Marshall et al. 2009 and Ray et al. 2010

Sensitivity= Ability of the test to identify patients who truly have the disease= $A/A+C$

Specificity= Ability to rule out the patients who truly do not have the disease= $D/B+D$

Positive predictive value= Likelihood that the disease is present in the case of a positive test result= $A/A+B$

Negative predictive value= Likelihood that the disease is absent in the case of a negative test result= $D/C+D$

Positive likelihood ratio (LR+) =Ratio of “true positive” measurements (=sensitivity) to “false positive” measurements (=1-specificity) = $sensitivity/1-specificity$

Negative likelihood ratio (LR-) = Ratio of “false negative” measurements (1-sensitivity) to “true negative” measurements (specificity) = $1-sensitivity/specificity$

Accuracy= Proportion of true results (true positives and true negatives) of all measurements= $A+D/A+B+C+D$

Sensitivity and specificity depend on the case mix and severity of the disease, and positive and negative predictive values are affected by the prevalence of the disease.

Likelihood ratios can be calculated for different levels of the test. A graphical plot reconstructed from the likelihood ratios of individual measurements produces the **ROC curve**. By calculating the **area under the (ROC) curve (AUC)**, the discriminative power of the test can be evaluated (Ray et al. 2010, Marshall et al. 2009). A rule of thumb for evaluating the performance of a biomarker in diagnostics or outcome prediction suggests the following limits: $LR+ > 10$ and $AUC > 0.90$ for excellent performance, $LR+ 5-10$ and $AUC 0.75-0.90$ for good performance, $LR+ 1-5$ and $AUC 0.50-0.75$ for poor performance and $LR+=1$ and $AUC 0.5$ for no diagnostic or predictive value (Ray et al. 2010).

3. AIMS OF THE STUDY

The role of neutrophil-derived matrix metalloproteinases and their inhibitors in different conditions with systemic inflammation, namely severe infection, sepsis and organ dysfunction, but also in ischaemia/reperfusion injury was investigated. Detailed aims of the study are described below.

1. To examine the systemic levels of MMP-7 (III), MMP-8 (I, II, III) and MMP-9 (II, III) and their inhibitor, tissue inhibitor of metalloproteinases-1 (TIMP-1) (II, III), in septic and non-septic groups of critically ill patients compared with healthy controls.
2. To evaluate the local levels of MMP-8 in an infected organ compartment and compare its local levels with its serum and urine concentrations in a patient group with secondary peritonitis (I).
3. To examine the association of systemic MMP-8 (II, IV), MMP-9 (II) and TIMP-1 (II, IV) levels with mortality in patients with severe sepsis or septic shock.
4. To evaluate MMP-8 and TIMP-1 in outcome prediction in a large group of patients presenting with acute respiratory failure and in a subgroup of patients with ALI/ARDS (IV).
5. To investigate the association of mild therapeutic hypothermia treatment with serum MMP-7, MMP-8, MMP-9 and TIMP-1 levels (III).

4. PATIENTS AND METHODS

4.1. PATIENTS

This study comprised four separate patient populations and a group of healthy volunteers. The patient group in Study I was collected solely for the purposes of this study and Studies II, III and IV were substudies of large multi-centre studies. Patient groups for individual studies are described in detail in Table 8 below.

Table 8. Study patients.

Study	Study I	Study II	Study III	Study IV
Setting	2 ICUs in a University Hospital	24 Finnish ICUs	From one centre (Helsinki) in a multi-centre study	25 Finnish ICUs
Study conduction time	9 months January 1- September 30, 2004	4 months November 1- February 28, 2005	3 years, 4 months March 1997- June 2000	8 weeks April 16- June 10, 2007
Design and name of the study	Prospective observational	Prospective observational. FINNSEPSIS	Retrospective laboratory analysis from a prospective randomized controlled study. HACA	Prospective observational. FINNALI
Primary inclusion criteria	Surgically confirmed secondary peritonitis	Severe sepsis or septic shock (ACCP/SCCM criteria)	Witnessed cardiac arrest of presumably cardiac origin (absence of pulse and spontaneous respiration)	Mechanical ventilation for more than 6 hours
Other inclusion criteria	Age >18 years	Age >18 years	-Age 18-75 years -VT/VF as initial rhythm -ROSC < 60 minutes -Time from collapse 5-15 minutes until resuscitation attempts by emergency medical personnel	Age >16 years
Exclusion criteria	-Metastasing malignancy -Haematological malignancy -HIV, immunocompromising disease, immunosuppressive medication -Tetracycline or bisphosphonate use		-CA due to intoxication or trauma -Collapse in the presence of medical personnel -Response to verbal command -Hypothermia (<30°C on admission) -Terminal illness, coagulopathy or pregnancy -Sustained hypotension or hypoxemia	-Immuno-compromised patients -Systemic corticosteroid use -Cytostatic medication
Inclusion time to study	<72 hours from surgery	When inclusion criteria first met	On arrival at the emergency department	After 6 hours of mechanical ventilation
Number of patients/ Consent to blood samples/ Total number of patients in the original study	15	248/ 254/ 470	51/70 Hypothermia 30/36 Normothermia 21/34	563/ 656/ 958

ICU= intensive care unit; VT= ventricular tachycardia; VF= ventricular fibrillation; ROSC= restoration of spontaneous circulation; CA= cardiac arrest

In Studies I and III, the inclusion of individual patients was assessed by the study investigators. In Studies II and IV, this was done by the treating clinicians on the basis of written criteria. Patients in Studies I, II, and IV received standard treatment in the ICUs. Patients in Study III were randomized to receive either mild therapeutic hypothermia with active cooling to 33 \pm 1°C for 24 hours or normothermia. Normothermia treatment included passive warming after possible spontaneous hypothermia on arrival to a body temperature of less than 38°C, where the temperature was then maintained. Hypothermia was induced with an external cooling device, and after 24 hours of hypothermia patients were rewarmed over an 8-hour period.

4.2. CONTROLS

Ten healthy controls with no medications served as a control group for blood (I, II, III) and urine (n=9) (I) samples. All controls gave informed consent before sample collection.

4.3. DATA COLLECTION

Data were collected manually in Studies I and III as well as partly in Studies II and IV. In Studies II and IV, intensive care diagnoses, clinical variables, severity scorings and organ failure scorings, and ICU mortality were obtained from the Finnish Intensive Care Consortium (Intensium Ltd., Kuopio, Finland). Hospital mortality in Studies I, II and III were assessed by investigators. Data considering 30-day and 90-day mortality were obtained from Statistics Finland (IV).

Demographic data, chronic illnesses and medications were recorded in all studies. Source of infection, microbiological data and antibiotic treatment were recorded (I, II). The aetiology of cardiac arrest was assessed by a specialist in cardiology. The presence of ALI/ARDS was determined by ICU clinicians and radiologists in the admitting hospital. Risk factors for ALI/ARDS were recorded (IV).

APACHE II (Knaus et al. 1985) (I, II) and SAPS II (LeGall et al. 1993) (II, IV) were used as measures of disease severity, and daily calculations of SOFA scores (Vincent et al. 1998) (I, II, IV) were used for organ failure assessment. In Study I Mannheim peritonitis index (Wacha et al. 1987) was calculated. Use of hydrocortisone in the treatment of septic shock was recorded in Studies I, II and IV. Laboratory parameters recorded included C-reactive protein (CRP), white blood cell (WBC) count, creatinine and platelet count, and in Study III neuron-specific enolase, S-100B-protein, troponin T (TnT) and creatinine kinase (CK) and its MB fraction (CK-MB).

4.4. DEFINITIONS

Severe sepsis and septic shock, defined by using the ACCP/SCCM criteria (Bone 1992), comprised systemic inflammatory response, suspected or confirmed infection and acute organ dysfunction, and in the case of septic shock, also persistent hypotension (II). Severe organ dysfunction or organ failure was pronounced when the SOFA score was 3 or more in an individual organ system (II).

Cardiac arrest was defined by the absence of arterial pulses and spontaneous respiration (III).

Acute respiratory failure was defined by the need for prolonged (>6 hours) mechanical ventilation either with non-invasive or invasive interface (IV). ALI and ARDS were defined by the AECC criteria (Bernard 1994) (IV). Septic shock was defined by the need for vasoactive medication in Study IV.

4.5. SAMPLES FOR MMP AND TIMP-1 ANALYSES

4.5.1 BLOOD SAMPLES

In Studies I, II and IV, informed consent was required for collection of blood samples. In Study III, the ethics committee had waived the need for informed consent and a deferred consent was used for all patients.

Time-points for sample collection were the day of inclusion (I, II), 24 and 48 hours after ROSC (III) and at inclusion to the study and 48 hours thereafter (IV). The blood samples in Study I were taken at the same time as urine and peritoneal fluid samples.

Blood samples were drawn from an arterial line or venous indwelling catheters into a vacuum glass tube. Blood samples from healthy controls were taken from a peripheral vein. The samples were allowed to clot at room temperature and then centrifuged. The supernatants were separated and divided into aliquots and stored at -18°C (III) to -20°C until analysis. The samples in Study III were stored initially at -18°C and then at -70°C until taken to the analysing laboratory.

4.5.2 URINE AND PERITONEAL FLUID SAMPLES

Urine (20 ml) was drawn from urinary catheters. Peritoneal fluid samples were taken from peritoneal drainage tubes placed on clinical indication in laparotomy. The urine and peritoneal fluid samples were placed on ice, and then centrifuged at 500 rpm. for 15 minutes to separate cells and debris and finally stored at -20°C until analysis.

4.6. LABORATORY ANALYSES

4.6.1. MEASUREMENT OF PERITONEAL FLUID COLLAGENASE ACTIVITY (I)

To demonstrate collagenase activity in the peritoneal fluid, the peritoneal fluid samples were incubated with 1.5 $\mu\text{mol/L}$ soluble human skin type I collagen for 12 hours at 22°C. This was done with and without 1 mmol/L aminophenyl mercuric acetate (APMA) (Sigma, St. Louis, MO, USA). The characteristic $\frac{3}{4}$ (αA , 75%) cleavage products were separated by 8% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (Hanemaaijer et al. 1997).

4.6.2. WESTERN IMMUNOBLOTTING (I)

Specific polyclonal antibodies (IgG fraction) against human neutrophil-type MMP-8 (Hanemaaijer et al. 1997) and against MMP-1 and MMP-13 (Lauhio et al. 1994^c, Lindy et al. 1997) were used. Samples were mixed with Laemmli buffer without reducing agents and heated for 5 minutes at 100°C, followed by protein separation by using 10% SDS polyacrylamide gels. Target enzyme detection was performed by using an enhanced chemiluminescence (ECL) Western blotting system according to the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, UK). The immunoblots were quantified by a Bio-Rad Model GS-700 Imaging Densitometer using the Molecular Analysis program (Bio-Rad Laboratories Inc., Hercules, CA, USA). Human rheumatoid synovial fibroblast culture medium was used as a positive control to distinguish neutrophil-type MMP-8 from fibroblast or mesenchymal-type MMP-8 as well as for MMP-1 and MMP-13 (Hanemaaijer 1997).

4.6.3. TIME-RESOLVED IMMUNOFLUOROMETRIC ASSAY (IFMA) FOR MMP-8 ANALYSES (I, III, IV)

The MMP-8 concentrations in serum (I, III, IV), peritoneal fluid and urine (I) were measured by using a time-resolved immunofluorometric assay, as previously described by Hanemaaijer et al. (1997). The monoclonal MMP-8 specific antibodies 8708 and 8706 (Medix Biochemica, Kauniainen, Finland) were used as a catching antibody and a tracer antibody, respectively. The tracer antibody was labelled using europium chelate (Hemmilä et al. 1984). The assay buffer contained 20 mmol/L Tris-HCl, pH 7.5, 0.5 mol/L sodium chloride, 5 mmol/L CaCl_2 , 50 $\mu\text{mol/L}$ Zn Cl_2 , 0.5% bovine serum albumin, 0.05% sodium azide and 20 mg/L diethylenetriamine penta-acetic acid. The assays were performed on microtitration plates in two steps. Twenty microlitres of samples and 80 μL of assay buffer with 2 $\mu\text{L/ml}$ normal

mouse serum were pipetted into the wells. After incubation, the wells were washed and filled with 100 μ L of assay buffer containing 8706-Eu-labelled antibody. After incubation and washing, 100 μ L of enhancement solution was added, and after 5 minutes, fluorescence was measured using a 1234 Delfia Research Fluorometer (Wallac, Turku, Finland). The levels of MMP-8 were expressed as μ g/L. For Study III, the levels of MMP-8 were expressed as ng/ml.

4.6.4. MEASUREMENT OF MMPS AND TIMP-1 BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The serum concentrations of MMP-7 (III), MMP-8 (II), MMP-9 (II, III) and TIMP-1 (II, III, IV) were measured by enzyme-linked immunosorbent assay (ELISA), as previously described by Rautelin et al. (2009). Based on manufacturer information, this method measures active, pro-, complexed and fragmented forms of MMPs and TIMP-1. Commercial kits were used for the determinations of concentrations (Biotrak ELISA System; Amersham Biosciences, Buckinghamshire, UK). For TIMP-1 in Study III DuoSet ELISA Development Systems (R&D Systems, Minneapolis, MN, USA) were used. The analyses were performed following instructions from the manufacturer in random order. The coefficients for intra-assay and interassay variability for the Biotrak ELISA System Kit are 2.1% and 2.4% for MMP-7, 2.5% and 4.1% for MMP-8, 8.2% and 9.9% for MMP-9 and 9.3% and 12.4% for TIMP-1, respectively. The corresponding detection limits are 0.16 ng/ml, 0.032 ng/ml, 0.6 ng/ml and 1.25 ng/ml.

4.7. OUTCOME MEASURES

In Study II, the primary outcome measure was ICU mortality. In Study III, the original design included neurological recovery as a primary outcome, measured by the Pittsburgh Outcome Scale (CPC) and 6-month survival as a secondary outcome, but these outcome measures were not used for the purposes of this study. In Study IV, the primary outcome measure was 90-day mortality.

4.8. STATISTICAL METHODS

The statistical analyses were performed by using SPSS 10.1. for Windows (SPSS Inc., Chicago, IL, USA) (I, III). For Study IV SPSS statistics version 20.0 (SPSS, Chicago, IL, USA) was used. Analyses for Study II were performed by using Graph

Pad Prism version 4 (Graph Pad Inc., San Diego, CA, USA).

Data were presented as median and interquartile range, absolute value and percentage, or mean and standard deviation (SD) / standard error of mean (SEM) as appropriate. $p < 0.05$ was considered significant, with the exception of correlation tests and logistic regression analyses, where $p < 0.01$ was considered significant. $p < 0.01$ was considered significant in Study III.

For comparisons of categorical data between groups, Fisher's exact test was used.

Due to the assumed non-normal distribution of the laboratory parameters, the Mann-Whitney U-test was used for comparisons of continuous variables between independent groups, and the Kruskal-Wallis test was used for comparisons of continuous variables between several independent groups. In Study II, comparisons between groups were also performed with unpaired *t*-test. For comparisons of laboratory variables within a group (I) Wilcoxon's signed-rank test was used.

In Study III, the comparison of laboratory variables between groups at two different time-points was performed by calculating changes over time in these variables and then calculating the differences between means (DIM) of these changes with 95% confidence intervals (Guyatt et al. 1994).

Stepwise multivariate logistic regression analysis was performed to study the independent effect of a variable on outcome (IV).

Receiver-operator characteristic (ROC) curve analysis was performed to assess the ability of a variable to predict outcome (IV). The optimal cut-off values were identified by finding the value of a variable with the best specificity and sensitivity. This was done by using the Youden method, identifying the point with the greatest distance on the ROC curve from the line of equality (Youden 1950). After identifying the cut-off values, the positive likelihood ratios (LR+) with 95% confidence intervals (95% CI) for the cut-off values in predicting outcome were calculated (IV).

The areas under the curve (AUCs) were calculated with 95% confidence intervals (IV). An AUC of 0.5 represents performance on the test equalling random chance, whereas a perfect test would yield AUC 1.

Spearman's *Rho* test was used to evaluate correlations between non-parametric variables (III, IV).

Kaplan-Meier survival curves were constructed according to quartiles of admission MMP-8 and TIMP-1 values (IV) to demonstrate the difference in 90-day mortality.

5. RESULTS

5.1. CHARACTERISTICS OF PATIENTS

The studies comprise a total of 877 patients. Characteristics of patients, clinical severity scores and outcome are summarized in Table 9.

Table 9. Patient and treatment characteristics, clinical severity scorings and outcome.

Study	I	II	III	IV
Study diagnosis	Secondary peritonitis	Severe sepsis or septic shock	Cardiac arrest	Acute respiratory failure
Number of patients	15	248	51	563
Age, years	63 (54-74)	60 (49-72)	59 (52-67)	64 (52-75)
Gender male	10 (66.7)	170 (70)	41 (80)	375 (67)
Need for vasoactive therapy	13 (86.7)	186 (75)	na	289 (56)*
Corticosteroid during study	5 (33)	71 (29)	2 (3.9)	na
WBC count (x10⁹/L) on study admission	8.1 (6.2-10.6)	na	11 (9-14.4)	10.5 (8-14.6)
CRP (mg/L) on study admission	263 (139-327)	na	5 (5-6)	51 (9-160)
Serum creatinine (μmol/L)	83 (67-113)	na	na	75 (57-117.5)
Patients with immunosuppressive medication	0	28 (11) corticosteroids 12 (5) cytostatic drugs	0	0
SOFA on admission	8 (5-9)	8 (6-9)	na	8 (5-10)
Highest SOFA	8 (5-9)	10 (8-13)	na	9 (6-12)
APACHE II	13 (8-17.5)	24 (18-29)	na	23 (16-29)
SAPS II	na	na	na	42 (29-55)
ICU mortality	na	33 (13)	na	52 (9)
Hospital mortality	2 (13.3)	62 (25)	10 (20)	116 (21)
90-day mortality	na	na	na	152 (27)

Values are presented as number (%) or median (interquartile range). * Vasoactive therapy on admission. WBC= white blood cells; CRP= C-reactive protein; SOFA= Sequential Organ Failure Assessment; APACHE II= Acute Physiology and Chronic Health Evaluation score II; SAPS II= Simplified Acute Physiology Score II; ICU= intensive care unit; na= not assessed.

In Studies II and III, the patients were comparable with the patients from the original studies without laboratory samples. Patients receiving immunosuppressive medications (n=61) were excluded from the final analyses in Study IV. A detailed description of disease characteristics, laboratory variables and therapy for Study III is provided in Table 10.

Table 10. Disease and treatment characteristics and laboratory variables in Study III.

Initial rhythm VF, n (%)	50 (98)
Basic life support, n (%)	21 (42)
Myocardial infarct, n (%)	31 (61)
Primary arrhythmia, n (%)	12 (24)
Thrombolysis, n (%)	20 (39)
Hypothermia treatment, n (%)	30 (59)
Time to ROSC, minutes, median (IQR)	17 (13-22)
Temperature on arrival to hospital, °C, median (IQR)	35.3 (34.7-35.9)
NSE at 24 hours, µg/L, median (IQR)	10.2 (7.7-13.5)
S100 at 24 hours, µg/L, median (IQR)	0.13 (0.1-0.17)
Troponin t at 24 hours, µg/L, median (IQR)	0.54 (0.09-1.71)

Data are presented as absolute values (%) or medians (interquartile range). VF= Ventricular fibrillation; ROSC= restoration of spontaneous circulation; NSE= Neuron-specific enolase.

The risk factors for acute respiratory failure (IV) for the study population and the ALI/ARDS subgroup are presented in Table 11.

Table 11. Type of admission and risk factors for acute respiratory failure in Study IV patients.

Condition within 48 hours of ICU admission	Non-ALI/ARDS patients (n= 520), n (%)	ALI/ARDS subgroup (n= 43), n (%)
Sepsis	61 (12)	12 (28)
Pneumonia	49 (10)	11 (26)
Infection	14 (3)	9 (21)
Cardiac insufficiency	111 (22)	11 (26)
Intoxication	33 (6)	5 (12)
Impaired consciousness	151 (29)	9 (21)
Neuromuscular disease	10 (2)	1 (2)
Suspected or confirmed aspiration	83 (15)	12 (28)
Pancreatitis	14 (3)	3 (7)
Trauma	34 (7)	1 (2)
Massive transfusion	33 (6)	3 (7)
Elective admission	95 (18)	0 (0)
Operative admission	214 (41)	7 (16)

Data are presented as absolute values and percentages. ICU= intensive care unit; ALI= acute lung injury; ARDS= acute respiratory distress syndrome.

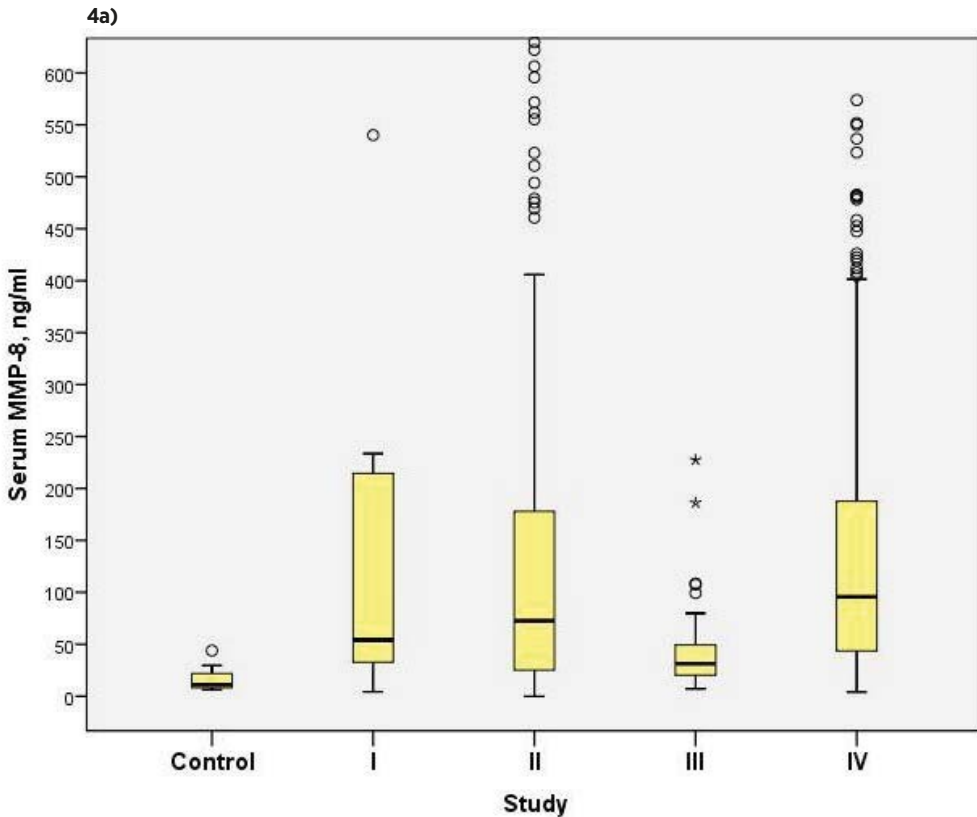
5.2. LEVELS OF MMP-8 IN SERUM AND BODY FLUIDS (I-III)

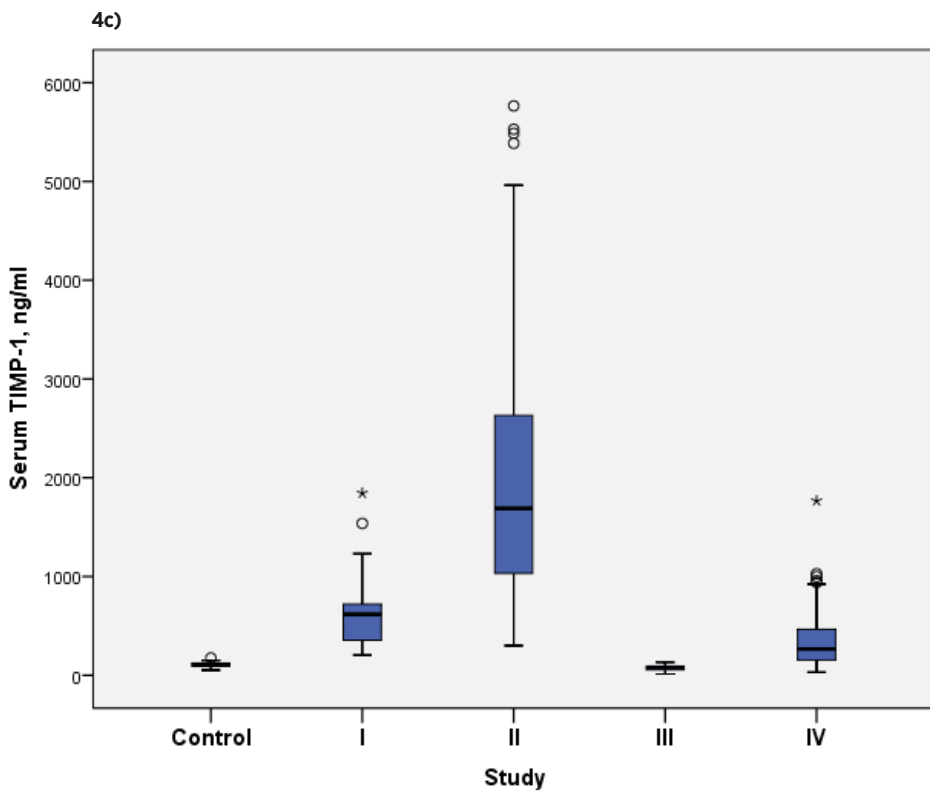
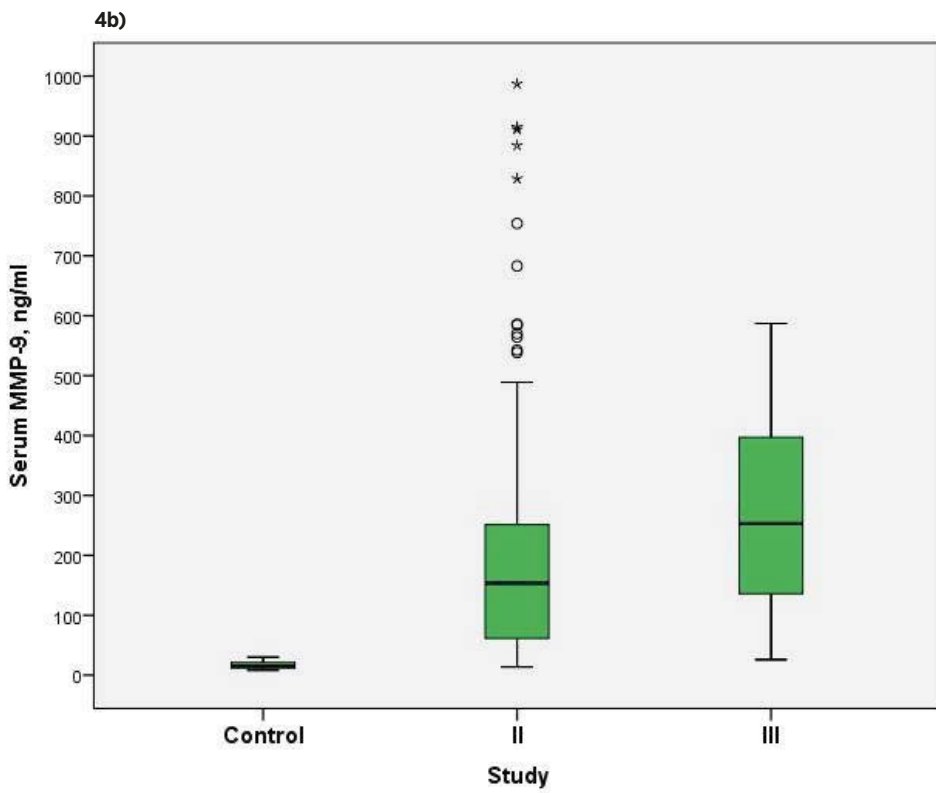
The serum levels of MMP-8 were significantly elevated in patients compared with controls (I-III) (error in study III, where a difference in MMP-8 is reported as not significant ($p=0.022$): it should be 0.002, and thus, is highly significant).

The MMP-8 levels in the sera of patients were 200-fold those in urine. The MMP-8 levels in peritoneal fluids were 25-fold those in sera. No correlations emerged between MMP-8 concentrations in different body fluid compartments. The peritoneal fluid was found to exert collagenolytic activity when incubated with human skin fibroblast type I collagen, and it was recognized to be due to neutrophil-type MMP-8 by immunoblotting (I).

The admission levels of serum MMP-8, MMP-9 and TIMP-1 in study patients and controls are shown in Figure 4.

Figure 4. Systemic MMP-8 (a), MMP-9 (b) and TIMP-1 (c) in the study patients and controls: a) MMP-8 levels were elevated in all study groups. b) MMP-9 was evaluated in patients with sepsis or septic shock (II) and in patients resuscitated from cardiac arrest. MMP-9 levels in study III appear higher than those in study II. c) Levels of TIMP-1 vary according to the study group, with the highest levels in patients with severe sepsis or septic shock.





5.3. SYSTEMIC LEVELS OF OTHER MMPS AND TIMP-1 IN DIFFERENT PATIENT POPULATIONS AND COMPARISON WITH CONTROLS (II-IV)

MMP-7 was slightly elevated in cardiac arrest patients relative to controls, but this was not significant at the level of $p < 0.01$ chosen for this study (III). MMP-9 concentrations were higher in patients than in controls (II, III). TIMP-1 levels, by contrast, were lower in cardiac arrest patients than in controls. These differences were highly significant.

5.4. CORRELATION OF MMPS AND TIMP-1 WITH CLINICAL AND LABORATORY VARIABLES (II)

MMP-8 correlated weakly with TIMP-1 (r^s 0.344, $p < 0.001$) and admission SOFA (r^s 0.208, $p = 0.001$), but not with APACHE II score in severe sepsis or septic shock patients. TIMP-1 correlated highly with maximal lactate on the first study day (r^s 0.569, $p < 0.001$), moderately with SOFA score (r^s 0.449, $p < 0.001$) and weakly with APACHE II score (r^s 0.351, $p < 0.001$) in these patients (unpublished results).

5.5. ASSOCIATION WITH OUTCOME (II, IV)

The association of MMP-8 (II, IV), MMP-9 (II) and TIMP-1 (II, IV) with mortality was investigated in severe sepsis or septic shock patients and in a large, unselected patient population with acute respiratory failure. Of the latter, 13% of the patients had sepsis on admission. When comparing admission MMP-8 levels between ICU survivors and non-survivors, significantly ($p < 0.01$) higher median MMP-8 levels were found in non-survivors, median 66.1 ng/ml (IQR 23.9 - 157.6 ng/ml) vs 149.6 ng/ml (IQR 41.8 - 477.1 ng/ml), respectively (II). Similarly, in acute respiratory failure patients, MMP-8 levels were higher in ICU non-survivors than survivors ($p = 0.013$). The difference in MMP-8 levels in Study II was no longer present when comparing hospital survivors and non-survivors (unpublished results). In Study IV, MMP-8 levels were similar between 90-day survivors and non-survivors. The ROC curve for MMP-8 and 90-day mortality is presented in Figure 5.

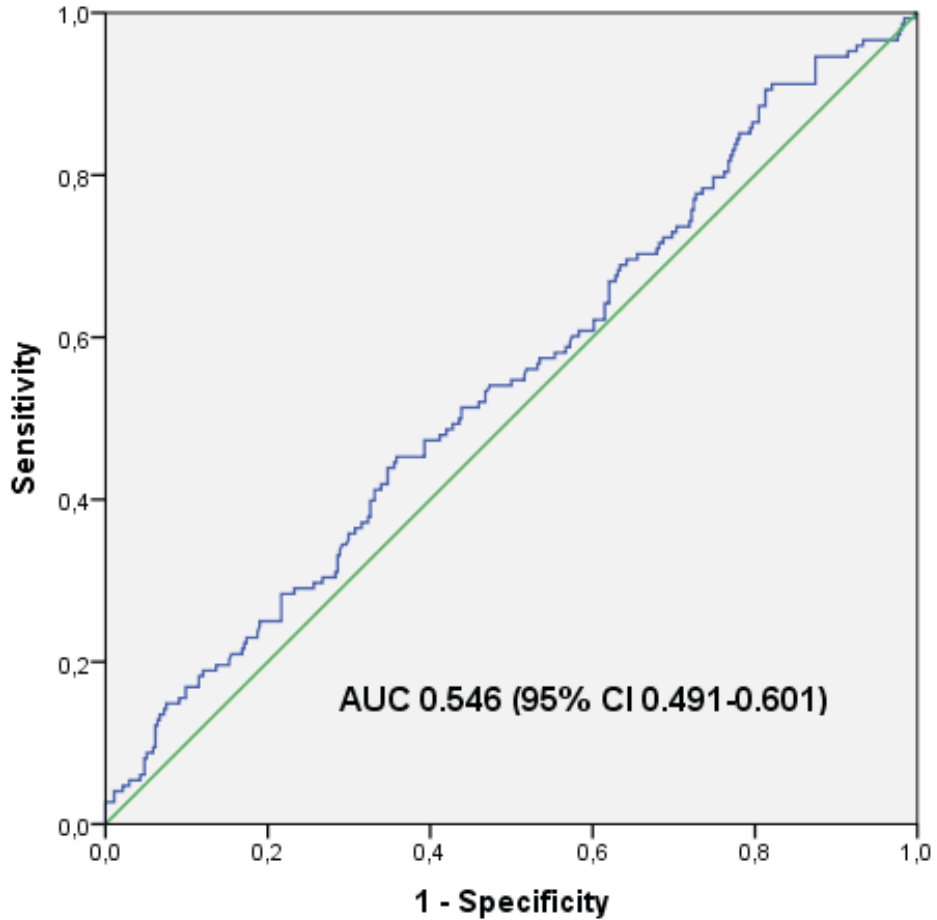


Figure 5. ROC curve: Performance of MMP-8 in predicting 90-day mortality in acute respiratory failure patients.

The area under the curve (AUC) was 0.546 (95% CI 0.491-0.601).

Median MMP-9 levels in ICU survivors, 158.7 ng/ml (IQR 66.5 - 251.9 ng/ml), and ICU non-survivors, 108 ng/ml (IQR 37.1 - 179.9 ng/ml), differed significantly ($p=0.047$), with lower values in non-survivors. The difference was no longer present when assessing 28-day mortality.

TIMP-1 levels were associated significantly with mortality in both studies. Figure 6 shows the comparison of admission serum TIMP-1 concentration between a) ICU survivors and non-survivors in Study II and b) 90-day survivors and non-survivors in Study IV.

5. RESULTS

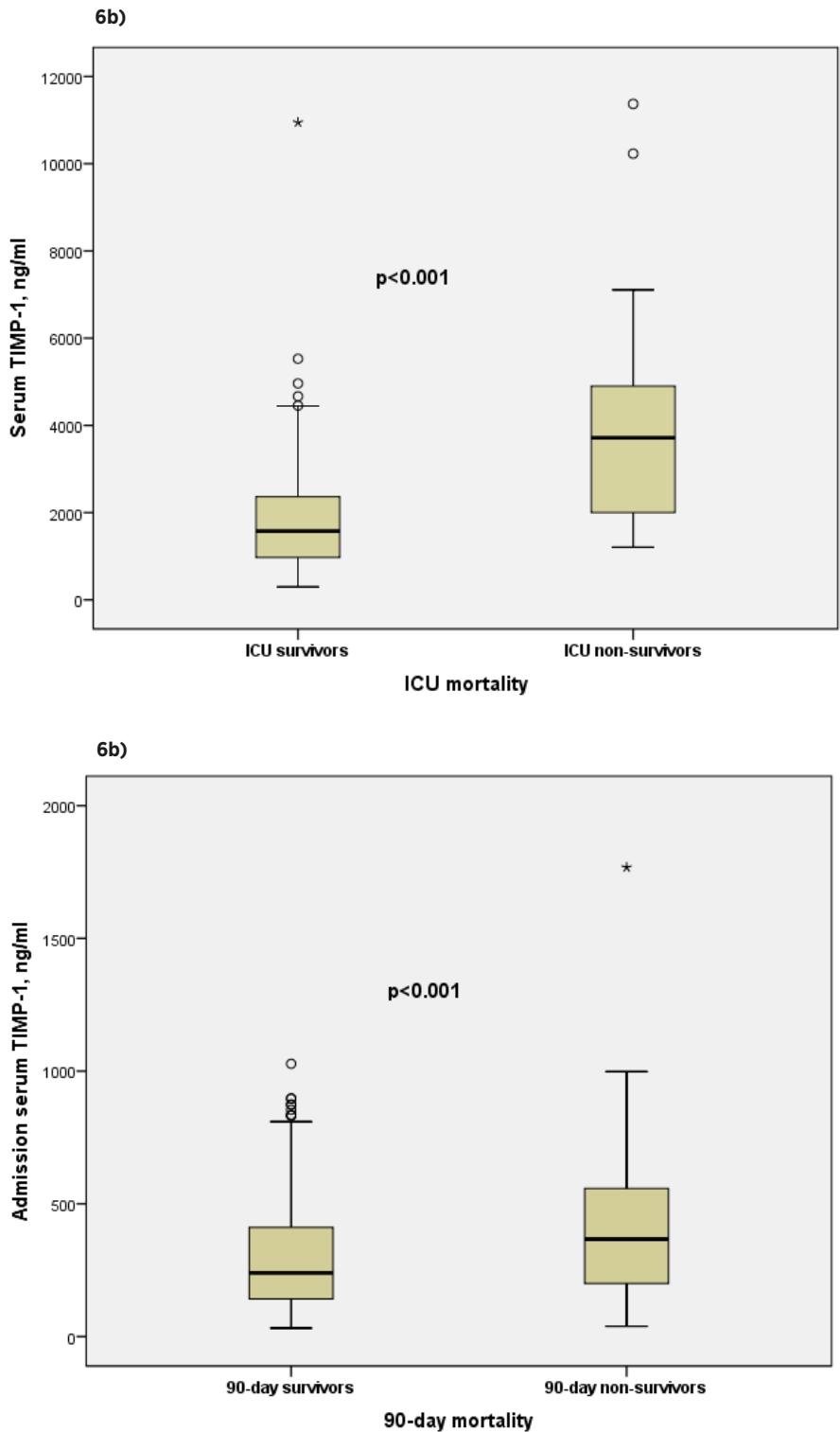


Figure 6. Comparison of admission serum TIMP-1 concentration between a) ICU survivors and non-survivors in Study II and b) 90-day survivors and non-survivors in Study IV.

TIMP-1 was an independent predictor of 90-day mortality and had an AUC of 0.633 (95% CI 0.580-0.686) in ROC analysis. This association was also present in elective patients included in the study, as shown in Figure 7.

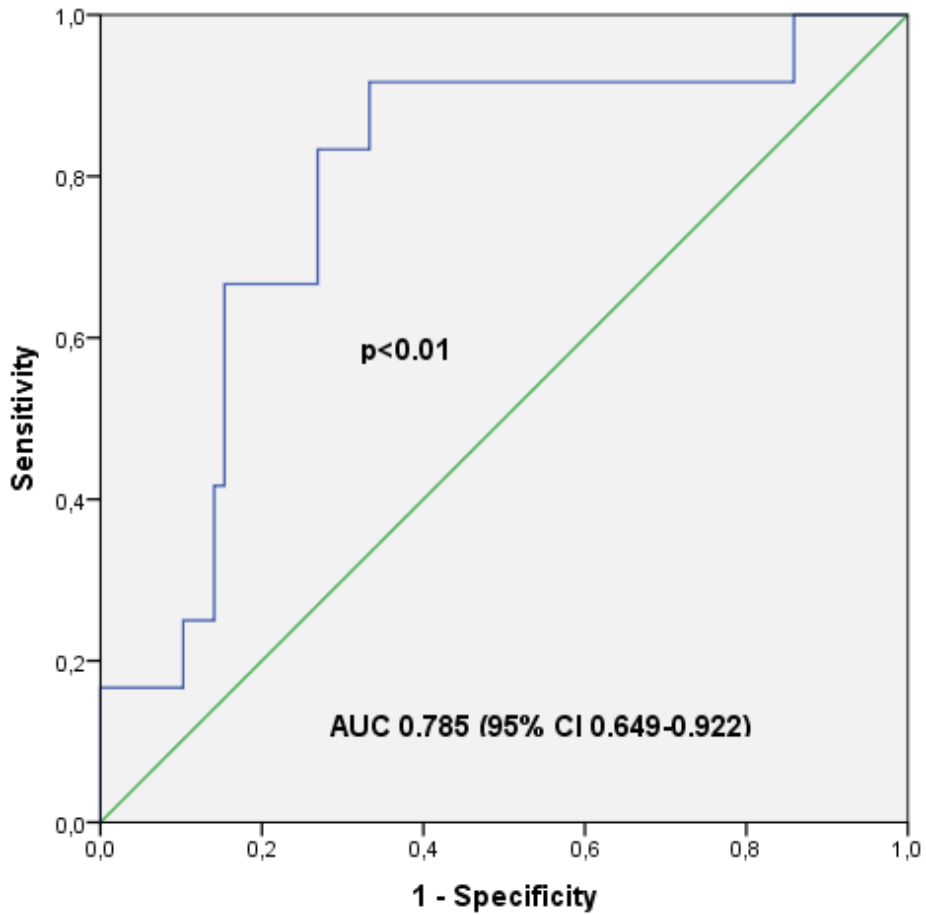


Figure 7. ROC curve: TIMP-1 in predicting 90-day mortality of acute respiratory failure patients with elective admissions.

5.6. ASSOCIATION WITH HYPOTHERMIA TREATMENT (III)

Patients who did (n=30) and patients who did not receive therapeutic hypothermia treatment (n=21) had differing MMP-9 levels in the first blood sample, which was taken 24 hours from ROSC. At this time, all patients in the hypothermia group had reached the target temperature. The comparison of serum MMP-7, MMP-8, MMP-9 and TIMP-1 values between the two patient groups is shown in Table 12.

Table 12. Comparison of serum MMP-7, MMP-8, MMP-9 and TIMP-1 values between hypothermia-, and non-hypothermia-treated patients at 24 hours from ROSC.

	MMP-7, ng/ml (IQR)	MMP-8, ng/ml (IQR)	MMP-9, ng/ml (IQR)	TIMP-1, ng/ml (IQR)
Non-hypothermia (n=21)	0.4 (0-1.9)	40.5 (27.1- 78.3)	372 (251.5-419.5)	77.4 (58.6-90.0)
Hypothermia (n=30)	0.5 (0.1-1.6)	24.2 (17.7-41.5)	147.5 (101.8-280)	81.6 (53.8-96.5)
p-value	0.69	0.02	0.001	0.48

Data are presented as median (interquartile range). $P < 0.01$ was considered significant. MMP= matrix metalloproteinase, TIMP= tissue inhibitor of metalloproteinases, IQR= interquartile range

In the second sample, taken at 48 hours, when none of the patients were hypothermic, no differences were present between the groups in any of the measured MMPs or TIMP-1. The change from 24 hours to 48 hours in MMP-9 concentrations between patient groups was significant ($p < 0.001$), whereas in MMP-7, MMP-8 and TIMP-1 the change was similar between groups. The median MMP-9 levels at 24 and 48 hours from ROSC for both groups are presented in Figure 8.

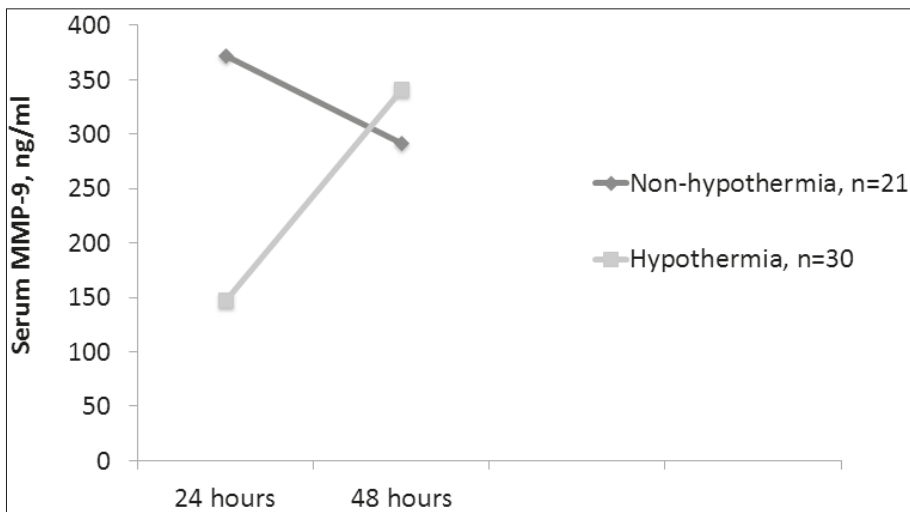


Figure 8. Median MMP-9 levels at 24 and 48 hours from ROSC for patients treated with hypothermia (n=30) and the patients with no hypothermia treatment (n=21). MMP-9 levels were lower in patients treated with hypothermia at 24 hours. The difference in changes over time (from 24 to 48 hours) was significant between groups.

5.7. MMP-8 AND HYDROCORTISONE TREATMENT (II)

Hydrocortisone was used to treat 33% (I), 29% (II) and 3.9% (III) of patients. In Study II, the association of hydrocortisone treatment with admission MMP-8 and -9 levels was examined. No difference was detected in the levels of either enzyme relative to the use of corticosteroids.

5.8. ASSOCIATION WITH OXYGENATION DISTURBANCE (IV)

MMP-8 levels had a weak negative correlation ($r^s = -0.162, p < 0.001$) with the degree of oxygenation disturbance defined by $\text{PaO}_2/\text{FiO}_2$ ratio. TIMP-1 ($p < 0.01$), but not MMP-8 ($p = 0.25$), was significantly higher in the ALI/ARDS subgroup than in other patients. The ability of TIMP-1 to predict 90-day mortality in patients with ALI/ARDS was moderate to good, with an AUC of 0.686 (95% CI 0.524-0.847, $p = 0.03$). At the cut-off level of 418 ng/ml, TIMP-1 had a positive likelihood ratio of 2.60 (95% CI 1.13-6.00) for 90-day mortality. TIMP-1 had a weak negative correlation ($r^s = -0.260, p < 0.001$) with the degree of oxygenation disturbance in the whole patient population. Figure 9 displays the TIMP-1 levels in the quartiles of $\text{PaO}_2/\text{FiO}_2$.

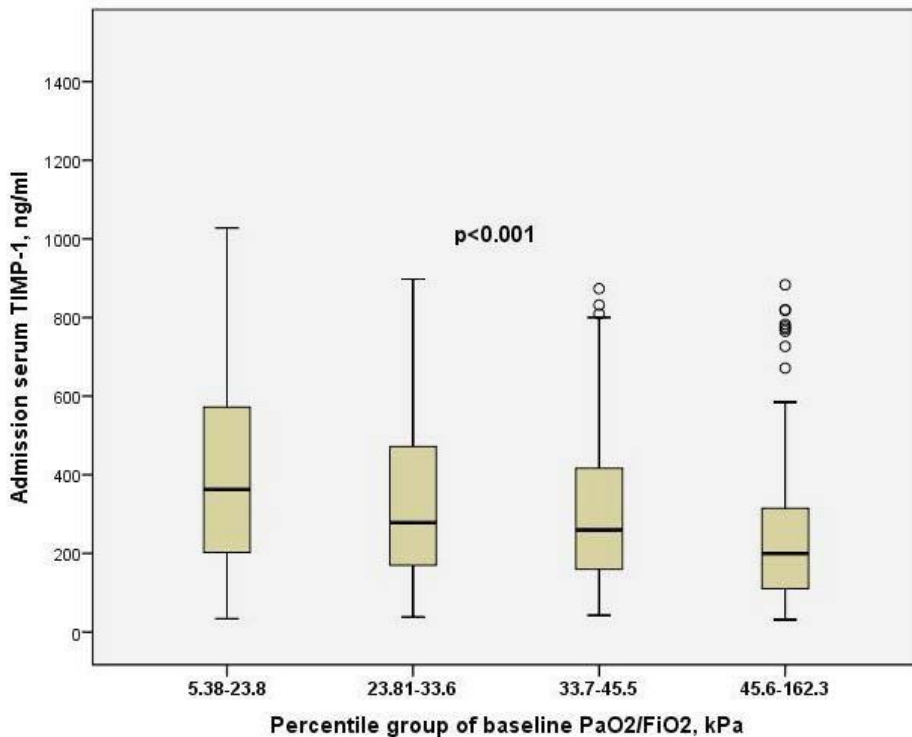


Figure 9. Admission serum TIMP-1 levels relative to oxygenation disturbance. The oxygenation disturbance is represented by the $\text{PaO}_2/\text{FiO}_2$ ratio from the 6 hours before inclusion in the study. The $\text{PaO}_2/\text{FiO}_2$ values for individual patients are divided into quartiles. The differences in TIMP-1 levels over the quartiles were significant.

5.9. POST HOC ANALYSES OF MMP-8, MMP-9 AND TIMP-1 IN PATIENTS WITH SEVERE SEPSIS OR SEPTIC SHOCK (II) AND ACUTE RESPIRATORY FAILURE (IV) (UNPUBLISHED RESULTS)

MMP-8 performed moderately in predicting ICU mortality, but not 28-day mortality of septic patients. TIMP-1 performed well in predicting ICU mortality and moderately in predicting 28-day mortality. The areas under the curve with 95% confidence intervals for admission MMP-8, MMP-9 and TIMP-1 in predicting ICU and 28-day mortality of patients with severe sepsis or septic shock are shown in Table 13.

At the cut-off-level of TIMP-1 2713 ng/ml, the LR+ was 3.82 (95% CI 2.57-5.66) for ICU mortality. The optimal cut-off value for TIMP-1 in predicting 28-day mortality was 1727 ng/ml. At this cut-off level, LR+ was 1.75 (95% CI 1.38-2.21).

Table 13. AUCs with 95% confidence intervals for MMP-8, MMP-9 and TIMP-1 in predicting ICU and 28-day mortality of patients with severe sepsis or septic shock. For comparison, the AUCs for APACHE II score and maximal lactate on day one are also shown (unpublished results).

	AUC, ICU mortality	95% CI	Significance	AUC, 28-day mortality	95% CI	Significance
MMP-8	0.644	0.539-0.750	0.008	0.572	0.489-0.654	ns
MMP-9	0.393	0.295-0.491	0.047	0.490	0.408-0.571	ns
TIMP-1	0.812	0.734-0.890	<0.001	0.687	0.608-0.766	<0.001
APACHE II	0.792	0.708-0.877	<0.001	0.704	0.624-0.784	<0.001
Maximal lactate	0.736	0.643-0.829	<0.001	0.701	0.620-0.782	<0.001

MMP=matrix metalloproteinase; TIMP= tissue inhibitor of metalloproteinases; APACHE II=Acute Physiology and Chronic Health Evaluation score II. AUC= area under the curve; ICU= intensive care unit; 95% CI= 95% confidence interval.

TIMP-1 was associated with organ dysfunction, as measured by lactate levels, which served as a surrogate marker for hypoperfusion. TIMP-1 correlated with the SOFA score on day one (r^s 0.449, $p>0.001$) (II), and elevated levels were associated with increased risk for renal replacement therapy (IV). Figure 10 shows the correlation of admission TIMP-1 levels with maximal lactate on day 1 of the study.

Figure 11 shows the ROC curve for TIMP-1 in predicting renal replacement therapy in patients with acute respiratory failure. The ability of admission TIMP-1 to predict renal replacement therapy was good, AUC 0.749 (95% CI 0.682-0.816) ($p < 0.001$).

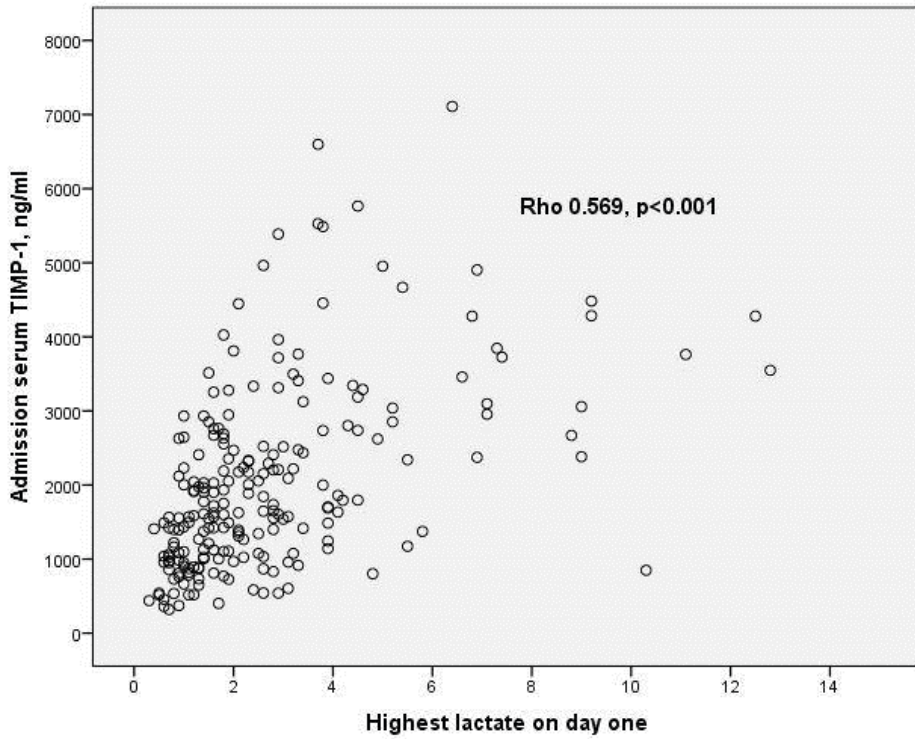


Figure 10. Correlations of admission TIMP-1 levels with maximal lactate on day 1 of the study (unpublished results).

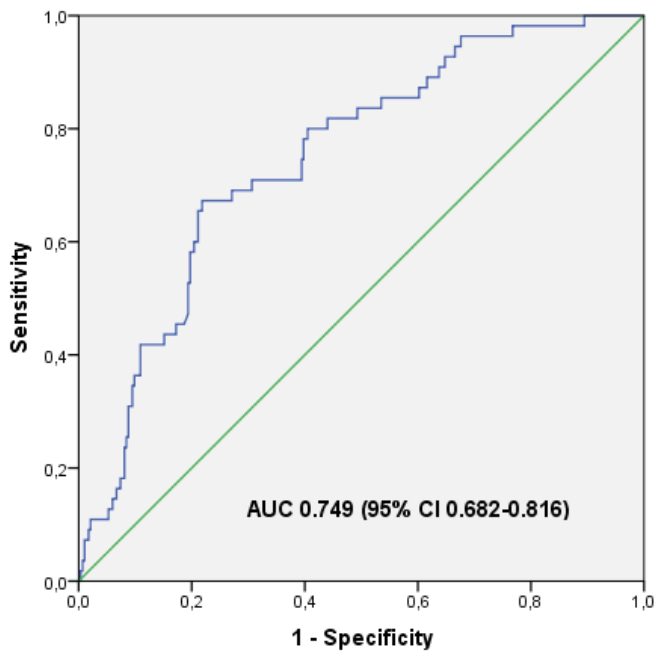


Figure 11. Admission TIMP-1 in predicting the risk for renal replacement therapy (n=56/ 371 with available information) in patients with acute respiratory failure (unpublished results).

6. DISCUSSION

Two decades ago MMPs were a subject of great interest in the field of cancer research, and initial experimental work led to clinical studies examining MMP inhibition. At the same time, it was discovered that these enzymes were important in chronic inflammation and had a potentially treatable harmful role in arthritis and periodontal disease. Results from clinical studies on cancer patients turned out to be disappointing, but in periodontal disease the MMP inhibitor doxycycline is currently an FDA-approved treatment. Advances in knowledge about MMPs led to spreading of the focus of interest to several fields, such as cardiovascular diseases, acute and chronic neurological diseases and pulmonary diseases. In the late 1990s to the early 2000s, the first clinical reports of neutrophil-derived MMPs in association with acute infection and inflammation were published. This study was then planned to describe the role of MMPs in critically ill patients with severe infection. In Study I, we then describe the presence of high levels of neutrophil-derived MMP-8 in the peritoneal fluid and other body fluids of critically ill patients with peritonitis for the first time. It was already known that the other neutrophil-derived MMP, MMP-9, is present in another type of peritonitis, associated with CAPD (Fukudome et al. 2001), and thus, the presence of MMP-8 was not unexpected. It was, however, a required step in increasing the knowledge of the role of MMPs in acute severe infections. Moreover, our study was among the first clinical studies reporting elevated MMP-8 levels in the systemic circulation of patients with severe sepsis or septic shock compared with healthy controls. This preliminary finding was corroborated in Study II. Because the patient population in Study I was small and the study was not planned to investigate the association of MMP-8 with mortality, this question was explored in the larger population of Study II.

6.1. MMP-8, MMP-9 AND MORTALITY

Many animal studies have shown that MMPs -8 and -9 are elevated in sepsis and septic shock and that inhibiting these enzymes pharmacologically may reduce sepsis-associated organ dysfunction and mortality. This is indirect evidence of the possibly detrimental role of these enzymes, but the evidence is far from compelling in human sepsis. In Study II, we found that MMP-8 levels were higher in patients who died in the ICU than in those who survived. This was a new finding that was recently indirectly substantiated by Solan et al. (2012) in a retrospective study on a paediatric population, where increased synthesis of MMP-8 (MMP-8 mRNA) was associated

with increased 28-day mortality. The association with mortality was, however, no longer present in our study population when examining 28-day mortality *post hoc*. Moreover, no association with mortality was found by Gäddnäs et al. (2010) at any time point in a smaller one-centre study with a patient population similar to ours. In our larger, albeit more heterogeneous, patient population consisting of patients with ARF (Study IV), MMP-8 was not associated with 90-day mortality. From a clinical point of view, this association with early, but not late, mortality may reduce the interest towards MMP-8 in sepsis for a couple of reasons. First, today's intensive care is effective and many of the patients who before succumbed to the initial shock phase now survive. Those who do not survive are often in full-blown shock on arrival to the ICU and the whole inflammatory cascade is fully activated, with death occurring rapidly despite maximal therapy. The patients who do survive this initial shock phase may either recover or enter a stage of sustained organ dysfunction and failure to recover due to "chronic critical illness" described by anorexia, loss of muscle mass, endocrinological changes and immunosuppression (reviewed by Marshall 2001 and Hotchkiss 2006). The latter is characterized by an inability to resolve the initial infection or susceptibility to secondary infections, often caused by low-virulence organisms. This group of patients comprises the largest number of patients dying of sepsis, not the group that succumbs to shock (reviewed by Hotchkiss 2006). In addition to unquestionably increased human suffering, this information is important in terms of health care resources. Therefore, ICU mortality is no longer a preferred end-point when assessing outcome, and a longer perspective is recommended (Bone et al. 1992). Second, in the search for biomarkers for outcome prediction, it is important to find a biomarker that brings added information to pre-existing tools and clinical assessment (Ray et al. 2010). In clinical practice, one of the most important questions is how can patients who are likely to develop organ dysfunction be found in time to prevent the detrimental pathophysiological chain of events. Of equal importance is to identify those patients who are unlikely to recover, to prevent futile care. In this respect, a biomarker like MMP-8 that can predict only short-term outcome may not be useful.

On the other hand, our results are interesting from the pathophysiological point of view. There are several possible explanations for why MMP-8 is associated with early, but not late, mortality. First, it is quite clear from experimental studies that MMP-8 participates in many stages of the inflammatory cascades. Therefore, it is hardly surprising that the levels of the enzyme are elevated relative to healthy controls in SIRS, the most extreme example of uncontrolled inflammation, a phenomenon demonstrated in all studies of this thesis. However, in studies of knock-out animals a certain pattern of inflammation is seen in MMP-8-deficient animals compared to WT animals, namely the delayed infiltration of neutrophils to the site of inflammation, but also a delayed resolution of the neutrophil infiltrate,

and thus, prolonged inflammation. This suggests that the role of MMP-8 is dual in sepsis: initially potentially harmful by causing tissue destruction and permeability changes associated with, for example, neutrophil transmigration, but later possibly required for the resolution of inflammation by, for instance, neutrophil apoptosis and in subsequent repair and remodelling processes. This might be one explanation for why high levels of the enzyme were associated with early mortality only. More information could certainly be gained with serial measurements of the enzyme during the course of the disease.

Another important issue is what we actually measure and how we do it. In most studies, MMP-8 and TIMP levels are measured by ELISA, which measures the total amount of the enzymes and TIMPs, both bound and unbound and in latent and activated forms. The same applies to the IFMA assay used for MMP-8 analyses in Studies I, III and IV. At the moment, no simple quantitative analysis method for the active form of the enzyme is available. Understanding the role of MMP-8 would be enhanced by such measurements because only the biologically active enzyme is likely to be relevant. MMPs are under strict control by several mechanisms. In the systemic circulation, their activity is inhibited by non-specific plasma proteins, such as α 2-macroglobulin, and evidence from several studies indicates that the systemic levels of TIMP-1 and possibly other TIMPs not measured are also elevated to a high extent. Given the simultaneous upregulation of other MMPs, like MMP-9 and others, that are also inhibited by the same proteins, it is very difficult to assess the amount of active enzymes in the circulation or locally. Of course, the high circulating levels may represent a pool of potentially activatable enzymes (Mühl et al. 2011), but all in all the interpretation of concentrations must be done cautiously. To circumvent this problem, some studies have used the ratio of MMP and TIMP as a surrogate measure of enzyme activity, but the above-mentioned overlap in the inhibitory function of TIMPs on MMPs makes this questionable. This critique is even more relevant considering studies using MMP-8 mRNA as a predictive tool.

Bearing these pitfalls of measurement in mind, an interesting finding from Study II was that non-survivors had lower serum levels of MMP-9 than survivors. Only one small, earlier study on septic patients has reported higher MMP-9 levels in non-survivors (Nakamura et al. 1998); as others have found no difference, or in one large study, lower levels in non-survivors (Lorente et al. 2009). This makes MMP-9 a poor biomarker candidate because its levels are low both in healthy controls and in individuals prone to die. Because inflammation evolved in order to help the host eradicate invading micro-organisms, one may speculate that a certain degree of MMP-9 upregulation is beneficial and the failure to express enzymes that help neutrophils to carry out this function could lead to worse outcomes. It would, however, be too simplistic to conclude that MMP-9 is merely protective in sepsis on the basis of these results, for several reasons. First, in two studies MMP-9 levels

were associated with measures of organ dysfunction (Lorente et al. 2009, Gäddnäs et al. 2010), although a negative correlation also has been reported (Yazdan-Ashoori et al. 2011). Second, many experimental studies again suggest that in MMP-9 knockout animals or by using an MMP inhibitor the outcome may be improved. Third, the cellular origin of MMP-9 is different from that of MMP-8. Whereas MMP-8 is mainly neutrophil-derived, various other cell types can be induced to produce MMP-9 by pro-inflammatory mediators. Thus, the initial fast release from neutrophils is probably followed by a secondary induction and upregulation at the tissue level, which may not yet (if ever) be visible in systemic samples taken during early phases at the ICU. This challenges us to study the time course of different MMPs and their inhibitors during ICU treatment and to search for a difference in change over time in concentrations between patients with favourable and unfavourable outcomes. Recently, Mühl et al. (2011) reported a study with serial measurements of MMP-2, MMP-9, TIMP-1 and TIMP-2 on five consecutive days in patients with severe sepsis, finding that MMP-9 levels were highest on the day of admission, thereafter declining. They did not, however, report any mortality data. In another study, Yazdan-Ashoori et al. (2011) found similarly high MMP-9 levels on day 1, with a subsequent decline. Especially in case of markers elevating very early, it should be noted that admission as a time-point for sampling is also a diffuse concept. Timing calculated from the beginning of fulfilment of SIRS criteria is somewhat more uniform. But even that depends on timing of examination, and we may never reach patients at exactly the same phase of the disease, with the exception of absolute events such as trauma, burns, cardiac arrest or elective operations. This restricts the information that can be gained by taking a single snapshot-like sample during a dynamic process like SIRS.

6.2. INHIBITION OF MMPS IN SEPSIS

Experimental studies almost uniformly show less tissue destruction, less organ dysfunction and an improved outcome by using an MMP inhibitor. This has also been the case with many other mediators, which have subsequently been tried in clinical trials with disappointing results. There are, of course, crucial differences between experimental animal models and human sepsis, beginning with differences between species and the poor equivalence of sepsis models, especially endotoxin models, with human infection. Importantly, the timing of inhibitor dosage is almost always very early, if not prophylactic, in these studies. This enables the inhibitor to exert its actions very early in the cascade, which is not the case in clinical reality. There are, however, at least three studies that showed a benefit from a later dosage of an MMP inhibitor (Steinberg et al. 2003, Meli et al. 2006, Leib et al. 2001). Moreover, the inhibitors are non-specific; for example, doxycycline inhibits both

MMP-8 and MMP-9 and TACE, thus diminishing also TNF- α levels. This may, however, also be an advantage when suppressing inflammation is the ultimate goal. Finally, the follow-up in experimental studies is almost always short and does not give us answers about the long-term effects of inhibitors.

The results of this study do not directly answer the question of whether MMP-8 and -9 are ultimately harmful or not. Given that they are elevated in the early phase of inflammatory response, have several potentially amplifying effects on inflammatory cascades and are associated with early mortality, a certain degree of inhibition in very severe shock could be useful. It has been suggested that doxycycline, which inhibits MMPs only partly, could be used in low sub-antimicrobial doses to eliminate the sharpest edge of inflammation, while still leaving the amount necessary for reparative functions (Sorsa et al. 2006). Since doxycycline is a well-known antibiotic with an acceptable safety profile and a proven effect in other patient groups (Gu et al. 2012), a randomized placebo-controlled double-blinded pilot study with early, very short-term, small-dose doxycycline is justified. One caveat in severe infection should, however, be kept in mind, namely the possible antagonism of a bacteriostatic and a bactericidal antibiotic. In 1951, Lepper et al. reported increased mortality when a tetracycline was combined with a beta-lactam antibiotic in treatment of pneumococcal meningitis in a study of 14 patients. Similar results were reported ten years later in another small study (Olsson et al. 1961). Recently, a beneficial effect of doxycycline as an adjuvant therapy with ceftriaxone was demonstrated in an experimental model of pneumococcal meningitis despite an *in vitro* partial antagonism of the antibiotics demonstrated in the same study (Meli et al. 2006). It might, however, be wise not to extend the pilot studies to include patients with pneumococcal meningitis.

6.3. TIMP-1 AND MORTALITY

In Studies II and IV, systemic levels of TIMP-1 were associated with increased mortality. TIMP-1 was included in the analyses because of its important role as an inhibitor of MMP-8 and MMP-9. The results were somewhat unexpected, considering the assumed harmful role of MMPs, not their inhibitors. There are, however, other findings in line with ours regarding patients with sepsis (Hoffmann et al. 2006, Lorente et al. 2009) as well as patients with acute lung injury (Ricou et al. 1996). Regarding ALI/ARDS, a low ratio of MMP-9/TIMP-1 in BAL was associated with more severe course of the disease (Lanchou et al. 2003, Ricou et al. 1996). Furthermore, TIMP-1 was independently associated with increased all-cause and cardiac mortality and with the incidence of myocardial infarction in a 24-month follow-up study of 389 male patients undergoing coronary angiography for variable reasons (Cavuosglu et al. 2006). It also predicted major adverse cardiac events in a large prospective observational study on myocardial infarction patients (Kelly et al. 2010). TIMP-1 is predictive of outcome also in some malignant diseases (Holten-Andersen et al. 2006, Nakopoulou et al. 2002). In Study IV the ability of TIMP-1 to predict mortality was fairly good also in electively operated patients needing mechanical ventilation for more than 6 hours. In Study II, the association was not analysed in detail, but in *post hoc* analyses the overall predictive ability was good, even better than that of the well-known APACHE II and SOFA scores and maximal lactate levels. This suggests that TIMP-1 should be further validated as a biomarker in predicting outcome and possibly separately in predicting dysfunction of different organ systems. In Study IV, a *post hoc* analysis, although limited in defining patient characteristics and organ dysfunction in detail, suggested that TIMP-1 levels predicted need for renal replacement therapy. TIMP-1 levels also correlated highly with lactate levels in our Study II population, suggesting an association with hypoperfusion or disturbances in oxygen delivery or utilization. Interestingly, in one study, TIMP-1 was associated independently with left ventricular systolic dysfunction (Cavuosglu et al. 2006). Moreover, TIMP-1 was associated with the degree of oxygenation disturbance in Study IV.

The explanation for the association of TIMP-1 with poor outcome may or may not lie in MMP inhibition; however this cannot be resolved by our results. TIMP-1 has been associated with increasing age (Holten-Andersen et al. 2006, Kelly et al. 2010), diabetes, impaired glomerular filtration rate, gender and hypertension (Kelly et al. 2010). TIMP-1 has also been shown to have functions that are independent of its MMP inhibitory capacity, such as erythroid potentiating activity, promotion of cell growth and pro-fibrotic functions (reviewed by Brew et al. 2010). It is also involved in steroidogenesis and is anti-angiogenic (reviewed by Lambert et al. 2004). In inflammation, it responds to both pro-inflammatory

and anti-inflammatory stimuli, depending on cell type (Marshall et al. 1993, Yao et al. 1997). An interesting feature of TIMP-1 with sepsis is its ability to activate neutrophils, inhibit their transmigration and inhibit neutrophil apoptosis (Chromek et al. 2004). This could hypothetically contribute to a prolonged inflammatory state, and thus, impaired prognosis. TIMP-1 is anti-apoptotic also for B-lymphocytes (Guedez et al. 1998). Understanding the pathophysiological significance of TIMP-1 in critically ill patients requires more detailed experimental studies, where the focus is on regulation of apoptosis of immune cells and pro-fibrotic mechanisms at the organ level. Evidence is accumulating that a high degree of immune cell apoptosis, particularly of T lymphocytes, is of great relevance in the development of a sepsis-associated immunosuppressive state and impaired prognosis (Hotchkiss et al. 1999).

6.4. MMPS AND TIMP-1 IN CARDIAC ARREST PATIENTS

The key findings in this study were the upregulation of MMPs -8 and -9 in cardiac arrest patients and the association of mild therapeutic hypothermia with lower levels of MMP-9. The elevated MMP levels in this patient group can be interpreted as further evidence of upregulated systemic inflammation as a part of the post-cardiac arrest syndrome pathophysiology, and the findings support the notion that non-infectious processes, such as ischaemia-reperfusion, may activate the metalloproteinase synthesis and/or release.

Our study was the first to describe the suppressive effect of hypothermia on MMP-9 in cardiac arrest patients. It gives more insight into the mechanisms underlying the beneficial effect of the treatment. Recently, Zhao et al. (2012) published similar results from a study using a swine model of cardiac arrest and MTH. They found that MTH was associated with lower serum levels of TNF- α , MMP-9 and NSE and suppressed mRNA expression of NF-kappaB, MMP-9 and aquaporin-4 in cortical tissues at 24 hours from ROSC. TIMP-1 was elevated in the hypothermia group, but no differences were found in IL-6 or IL-10 between groups. Their study also found no differences after hypothermia at 48 hours from ROSC. Interestingly, TIMP-1 has been shown to exert neuroprotection after glutamate excitotoxicity *in vitro* by a mechanism apparently independent of its MMP inhibitory function (Tan et al. 2003).

Because the samples analysed in our study were obtained from a study not originally designed for investigating MMPs, there are several possible confounding factors. MMP levels in our study may have been affected by the underlying cardiac disease or treatment. MMPs, MMP-8 and MMP-9 in particular, are important proteinases associated with atherosclerosis and destabilization of atherosclerotic plaques (Herman et al. 2001). Elevated systemic MMP-8 (Kato et al. 2005) and

MMP-9 levels are present in stable coronary artery disease, acute coronary syndromes (Blankenberg et al. 2003) and subsequent left ventricular dysfunction (Squire et al. 2004). Elevated MMP-8 (Tuomainen et al. 2007) and MMP-9 (Blankenberg et al. 2003, Hansson et al. 2011) are associated with an increased risk of cardiovascular death. Furthermore, the thrombolytic agent tPA is an MMP-9 activator (Horstmann et al. 2003). Smokers have higher MMP-8, TIMP-1 (Tuomainen et al. 2007) and MMP-9 levels, whereas statin users have lower levels (Blankenberg et al. 2003). Moreover, the patients may have had underlying acute or chronic infections that affected the MMP levels. On the other hand, CRP levels were generally very low. Considering these possible confounders, our results should be interpreted mainly as hypothesis-generating material.

6.5. SYSTEMIC MMP-8 IN ACUTE RESPIRATORY FAILURE

Acute respiratory failure patients and ALI/ARDS patients in particular were chosen to represent a diagnostic group of patients with severe inflammation and permeability changes in the pathophysiology of the disease. The evidence regarding MMP-9 involvement in lung injury pathophysiology was moderate in the literature, and thus, the failure of the other neutrophil-derived enzyme MMP-8 as a biomarker was somewhat disappointing. One explanation for the failure could be that the systemic levels of MMP-8 reflect local processes poorly. In fact in Study I we already found that the levels of MMP-8 in the infected compartment correlated poorly with systemic levels, but the study was too small to form firm conclusions. Most studies have examined the role of MMPs in lung injury in BAL fluids. Because performing BAL is not a simple procedure suitable for screening, we chose to evaluate systemic levels in this large study population. This was the first time that systemic MMP-8 has been investigated in acute respiratory failure patients. Some studies have examined systemic levels of MMP-9 (Pugin et al. 1999, Ricou et al. 1996). Attempting to correlate BAL fluid enzyme levels with systemic levels would be questionable due to the dilutional effect and uneven gain of lavage fluid. This is a difficulty in interpreting studies using BAL in general. The heterogeneity of the patient population with differing background diagnoses is another explanation. Not all patients would have necessarily filled the SIRS criteria. Further studies should concentrate on a well-defined diagnostic group of ALI/ARDS patients with systemic inflammation as an aetiological factor. A third explanation could be, as in Study II, that MMP-8 has a dual, U-shaped relation to mortality.

6.6. STRENGTHS AND LIMITATIONS OF THE STUDY

The strengths of this study lie in results that are novel and create new hypotheses of the role of MMPs and especially TIMP-1 in critical illness. Clinical research on MMPs and TIMPs is emerging, and our studies are among the first for these diagnostic groups. There are, however, several limitations of this study that need to be addressed.

The sample size in Study I was small. Because no previous studies existed, a power analysis was not performed, and the sample size was based on convenience (>10 patients). A larger sample size could have strengthened the results. The timing of the samples was relatively late in the course of the disease, and serial measurements of MMP-8 during the course of the disease would have been valuable. Furthermore, analysing other MMPs and TIMP-1 and other inflammatory mediators would have given more insight into the role of MMPs in secondary peritonitis. However, this design allowed us to describe a new phenomenon, which was the original purpose of the study. In Study II, instead of a single measurement, serial measurements of the variables would have enhanced our understanding of the dynamics of MMPs and TIMP-1 in severe sepsis and septic shock. Development of MMP and TIMP-1 levels over the course of the disease could have better helped us to assess the association with outcome. Furthermore, linking our variables to organ dysfunction in specific organ systems would have been of value. This is a subject for future studies that should also include other markers of inflammation in analyses. Another weakness of this study is that the statistical analyses were not planned to examine the predictive power of our variables, but concentrated on the comparison of variables' levels between survivors and non-survivors. Furthermore, hospital mortality or, rather, 90-day mortality should have been preferred as a primary outcome measure. Last, we used the same control group as in Study I, which is a limitation because the storage time of the samples was thus different.

Study III was small and retrospective. On the other hand, it would probably not be possible today to design a prospective study with a non-hypothermia-treated control group because of the wide implementation of MTH treatment and the level A evidence of its benefits. This makes the HACA samples invaluable regardless of the limitations. The most apparent weakness in this study was the lack of baseline blood samples. Therefore, the possibility that the variables' levels may have been different at baseline, before the hypothermia treatment, cannot be excluded. Because the samples were taken at time-points calculated from ROSC, the duration of hypothermia treatment at the time-points used may have varied. Furthermore, samples from all patients from the original study were not available. Thus, although the patients were similar when tested, they may have been different in some respect. The relatively long storage time before analysis may also have affected

our results, although the difference between groups would be expected to be smaller in that case. The sample size was too small for any analyses considering outcome. These limitations render the results of this study only hypothesis-generating and preliminary. In Study IV, the broad definition of acute respiratory failure led to inclusion of a wide variety of diagnostic groups that did not necessarily suffer from an inflammatory condition either systemically or locally. The subgroup of ALI/ARDS patients was small and the diagnosis was defined by the treating clinicians. These limitations may have biased our results and weakened the possibility of interpreting our findings. Another limitation is the restricted number of sampling time-points and the lack of analyses of other MMPs or inflammatory parameters.

The group of healthy controls was small and the controls were younger than the patients in all studies.

6.7. PRE-ANALYTICAL CONSIDERATIONS

Measuring MMPs, particularly MMP-9, in serum samples has been questioned in recent literature because the serum levels of the enzyme are higher than when using anticoagulated blood. This has been explained by a release of MMP-9 from neutrophils or activated platelets during the sampling process or coagulation (Makowski et al. 2003). The lowest MMP-9 levels are found when using citrated plasma (Makowski et al. 2003). The difference between plasma and serum is evident when comparing the proportion of active enzyme, but when measuring the total amount of the enzyme (pro- and activated form), the levels are correlated between each sampling system (Gerlach et al. 2007), and thus, within one study the use of any method can be considered acceptable. However, in future studies, use of citrated plasma is preferable. Additionally, freeze-thaw cycles of seven or more affect the enzyme levels (Souza-Tarla et al. 2005). In our studies, all the analyses were performed on first-thaw samples. The time between sample-taking and centrifugation did not significantly affect the MMP levels in a study by Gerlach et al. (2007).

6.8. ETHICAL CONSIDERATIONS

All of the study protocols were approved by their respective ethics boards. Informed consent was obtained for sample taking (I, II, IV). For Study III, a deferred consent was used in the original study, and all patients or their legal surrogates accepted the protocol and sample-taking. All patients received standard care during the studies (I, II, IV). In study III, the patients received standard or what was at that

time experimental therapy, which later was confirmed to be beneficial. The study protocols (I, II, IV) consisted of sample collection only. The sample collection likely did not cause discomfort, pain or harm to the patients because pre-existing lines or tubings were used. These studies can therefore be considered ethically acceptable.

6.9. FUTURE ASPECTS

Despite increasing interest, only a few clinical studies describing the course of MMPs and their inhibitors in different infections exist. Well-designed studies are needed to demonstrate the presence and time course of MMPs and TIMPs and their relationship to other inflammatory parameters in various diagnostic groups such as meningitis, pneumonia and peritonitis. Diagnostic implications are possible. Based on the apparently poor correlation of systemic and local levels of MMPs, these studies could primarily focus on measuring enzyme levels at the compartment level. Cerebrospinal fluid MMP levels could be compared between patients with viral and bacterial meningitis and patients with a non-infectious indication for CSF sampling. Demonstration of differing levels between groups could lead to development of a test to differentiate bacterial infection from other conditions. The advantage in MMPs -8 or -9 in this context is their rapid upregulation in inflammation. Thus, an MMP test could aid in prompt selection of a therapy. To be advantageous, detecting MMPs would presuppose a rapid quantitative or semi-quantitative test. Indeed, a rapid (within 15 minutes) quantitative chair-side test for detecting MMP-8 in periodontitis patients has been developed, and the results are in line with traditional assays such as IFMA (Sorsa et al. 2010.) In addition to odontology, the applicability of this analytical method could be tested in other settings as well. Analyses of body fluid MMP-8 levels could be performed with IFMA and the bed-side test could be applied to examine the comparability of the methods and then test the sensitivity and specificity of the tests against the gold standard. An analogous study could be planned for intubated patients with suspected VAP, where MMP levels in tracheal aspirates have been suggested to differentiate true VAP from non-VAP (Wilkinson et al. 2012). First, MMP-8 (and -9) could be determined and compared with a patient group without VAP, e.g. intubated operative patients. Then, as in suspected meningitis, a bed-side test and IFMA could be compared. If comparable, the bed-side test should then be validated against a gold standard in a different patient population. However, the determination of a reliable gold standard in diagnosing VAP is pending. The diagnostic approach could be further expanded to peritonitis patients to, for example, detect infection in the ascites fluid in patients with cirrhosis of the liver.

A randomized controlled double-blinded pilot study on the effects of an MMP inhibitor doxycycline on severe sepsis patients should be designed. Such a study

should examine also the pharmacokinetics of intravenous doxycycline in this patient group. Critically ill patients have an increased volume of distribution, and thus, the subantimicrobial dose sufficient to suppress MMP-levels is to be determined. A level of $<1 \mu\text{g/ml}$ has been accepted as subantimicrobial in other studies (Gu et al. 2012), where the oral route of administration has been used. Using the intravenous route, the dose is unknown. Plasma levels of MMPs and doxycycline should be measured serially during and after a short-term intervention of 3-5 days and the MMP levels in treatment and placebo groups compared. All adverse events should be carefully registered. If a dosage with acceptable safety levels and a measurable effect on MMP concentrations is found, a larger RCT should be designed to investigate the association of doxycycline treatment with such outcome measures as survival and development of organ dysfunction.

Today little is known about the role of MMP-8 and -9 in the development of sepsis-associated organ dysfunction. Recently, MMP-8 was suggested to predict the development of sustained acute kidney injury (Basu 2011). A good early marker for the risk of developing AKI is needed in order to stratify patients to clinical studies and treatment protocols. In search of such a marker, MMPs should be measured in plasma and urine of ICU patients and their ability to predict AKI evaluated. On the basis of our preliminary, unpublished analysis in Study IV patients, also TIMP-1 could be a promising biomarker for predicting AKI. Experimental evidence of the involvement of MMP-8 and -9 in the physiology of coagulation and fibrinolysis encourages studies analysing MMP levels in association with DIC.

MMPs -8 and -9 are currently being studied in a patient group characterized by massively increased capillary permeability and a well-defined time of injury, namely patients with severe burns. The hypothesis is that MMPs contribute to the increased permeability and shock associated with severe burns. Plasma, urine and, if present, blister fluid MMP levels are measured. Patients with severe burns exceeding 20% of the total body surface area are compared with patients with less severe burns and healthy controls, and the association of MMP levels with organ dysfunction is analysed. The ultimate goal of studies in this patient group is a subsequent study of MMP inhibition, but further planning requires detailed characterization of the role of MMPs in injury. Also their role in reparative processes and wound healing must be characterized.

Another diagnostic group with a limited amount of studies and a likely MMP-8 and -9 involvement is severe acute pancreatitis (SAP). New studies are needed because the treatment of SAP remains exclusively supportive. First, high levels of MMPs in acute pancreatitis patients should be demonstrated, comparing the patients with either healthy controls or patients with less-severe, self-limiting pancreatitis. The patient group with SAP should be sufficiently large to allow at least preliminary analysis of disease severity and outcome. If an association of high MMP-8 or -9 levels with increased mortality is found, this patient group could be

suitable for studies on MMP inhibition on the basis of existing evidence of benefit in experimental studies. Furthermore, in pancreatitis, no infection is present in the early phase, and thus, interfering with inflammation should not pose problems with impaired bacterial clearance in the early phase.

In the future, serial measurements over the course of ICU treatment of MMP-8 and MMP-9 in cardiac arrest patients should be performed and the association with outcome assessed in a large prospective patient population. If evidence of a deleterious role is confirmed, this patient group could be a subject of further studies on MMP inhibition as well. In experimental stroke studies, an MMP inhibitor produces additive benefits to hypothermia treatment in terms of decreased markers of apoptosis (Liu et al. 2008). In another study on experimental stroke, minocycline, which is a lipophilic tetracycline group antibiotic with MMP-inhibiting activities, reduced infarct size and permeability changes as effectively as hypothermia treatment (Nagel et al. 2008). The effects of minocycline compared with hypothermia treatment should be examined also in an experimental cardiac arrest model. After this, a pilot RCT comparing the combination of minocycline and standard treatment with standard treatment alone could be designed to evaluate the potential synergistic effect of the treatments.

More experimental studies examining the role and regulation of TIMP-1 in the pathophysiology of pro- and anti-inflammatory reactions are needed. It is unclear whether the high levels of TIMP-1 in sepsis are adaptive, or whether TIMP-1 has a detrimental role. The association of TIMP-1 with dysfunction of different organs could be scrutinized in a patient population with severe sepsis to find out which factors are associated with elevated TIMP-1.

To overcome the shortcomings of the traditional definition of SIRS and sepsis, a novel approach to sepsis, the PIRO model, has been proposed (Levy et al. 2003). PIRO attempts to create a staging, analogous to the established TNM staging used in oncology. The purpose of PIRO is to better stratify the patients to groups according to their predisposition (P), type of infection or insult (I), response (R) and organ dysfunction (O) (Levy 2003). The first study to create and validate a PIRO-based staging tool reported equivalence of the PIRO model to the traditional severity scorings (Rubulotta et al. 2009). However, in their model, no biomarkers were used. The authors suggested that the model should be refined in the future by incorporating genomic information in the “P” and biomarkers in the “R” components (Rubulotta et al. 2009). Clinical studies should focus on examining whether measuring TIMP-1 adds to the present models of outcome prediction. They could aim at creating new panels composed of TIMP-1 and other novel biomarkers to be incorporated in PIRO or other staging tools. Thus far, no individual biomarker in sepsis has proven superior to another. Within the FINNSEPSIS study, several biomarkers have been examined, such as VEGF (Karlsson et al. 2008), procalcitonin (Karlsson et al.

2010), N-terminal pro-brain natriuretic peptide (Varpula et al. 2007), cell-free DNA (Saukkonen et al. 2008), chromogranin A (Røsjø et al. 2012) and high-sensitivity troponin T (Røsjø et al. 2011). Compared with the results of these studies, TIMP-1 in our study shows the best performance in predicting ICU mortality. Of the biomarkers tested in the FINNSEPSIS population, chromogranin A has the best AUC in predicting hospital mortality (Røsjø et al. 2012). In other patient populations, IL-6 (Pettilä et al. 2002) and suPAR (Koch et al. 2011) have shown promising results, although IL-6 was not an independent predictor of mortality (Pettilä et al. 2002). In comparing our results with other studies on the FINNALI patients, TIMP-1 in our study performed superiorly to suPAR (Jalkanen et al. 2012), comparably to plasma cell-free DNA (Okkonen et al. 2011^a), and inferiorly to N-terminal pro-brain natriuretic peptide (Okkonen et al. 2011^b) in predicting 90-day mortality. In a recent study, Calfee et al. (2011) created and validated a panel of biomarkers for predicting mortality in acute lung injury. They found that a panel of admission values of five biomarkers, including ICAM-1, von Willebrand factor, IL-8, soluble tumour necrosis factor receptor-1 and surfactant protein-D, improved diagnostic accuracy when added to APACHE III, compared with APACHE III alone. The usefulness of creating a panel of the moderately performing biomarkers derived from the FINNALI study, including TIMP-1 should be examined in a clinical study.

A new era in the search for potential new biomarkers has already begun with the novel microarray and proteomics techniques that are able to screen a large number of substances upregulated in inflammation. However, research is needed to fill in the many gaps in our knowledge of the complex interactions between the inflammatory mediators, their regulators and inhibitors.

7. CONCLUSIONS

1. Systemic levels of MMP-8 and MMP-9 are elevated in various groups of critically ill patients compared with healthy controls, regardless of whether the initiating event of inflammation is of infectious or non-infectious origin. Serum MMP-7 levels are not significantly elevated in cardiac arrest patients. TIMP-1 is elevated in severe sepsis or septic shock patients, but reduced levels are seen after cardiac arrest compared with healthy controls.

2. High levels of mainly neutrophil-derived MMP-8 are present in the peritoneal fluid of patients with secondary peritonitis after surgery. The levels of MMP-8 in peritoneal fluid were higher than the corresponding levels in the serum and urine.

3. Elevated systemic MMP-8 and TIMP-1 are associated with increased ICU mortality in patients with severe sepsis or septic shock. Among these patients, MMP-9 levels are lower in ICU non-survivors than in ICU survivors.

4. Systemic MMP-8 is not useful in predicting 90-day mortality in patients with acute respiratory failure. TIMP-1 is a potentially useful biomarker with a moderate discriminative power in predicting 90-day mortality in acute respiratory failure patients.

5. Serum MMP-9 levels are lower in patients treated with mild therapeutic hypothermia than in patients receiving non-hypothermia treatment. Levels of MMP-7, MMP-8 and TIMP-1 did not change significantly in the hypothermia-treatment group compared with controls.

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